

VOL. X, No. 4

APRIL 1959

PLANT AND SOIL

International Journal of Plant Nutrition,
Plant Chemistry, Soil Microbiology and
Soil-borne Plant Diseases

JUN 23 '59

UNIVERSITY OF HAWAII
LIBRARY



MARTINUS NIJHOFF — PUBLISHER — THE HAGUE

PLANT AND SOIL

International Journal of Plant Nutrition, Plant Chemistry,
Soil Microbiology and Soil-borne Plant Diseases

Revue internationale de nutrition des plantes, de chimie végétale,
de microbiologie du sol et des maladies des plantes en rapport
avec le sol

Internationale Zeitschrift für Pflanzenernährung, Pflanzen-
chemie, Bodenmikrobiologie und bodenbedingte Pflanzenkrank-
heiten

Issued under the auspices of the

"Koninklijk Genootschap voor Landbouwwetenschap"
(Royal Netherlands Society of Agricultural Science)

Executive Editors: D. I. Arnon, Berkeley; J. Baeyens, Louvain;
F. C. Gerretsen, Groningen; G. W. Harmsen, Groningen; E. G.
Mulder, Wageningen; F. J. Richards, London; A. C. Schuffelen,
Wageningen; F. Steenbjerg, Copenhagen. **Secretary:** G. H. Arnold,
Groningen.

Consulting Editors: Wm. A. Albrecht, Columbia, Mo.; C. Arnaud, Milan;
E. Gäumann, Zurich; L. Gisiger, Liebefeld-Berne; F. G. Gregory, London;
A. J. de Groot, Groningen; J. den Holder, Ghent; T. H. van den Honert,
Leyden; V. J. Koningsberger, Utrecht; J. Lavollay, Paris; W. J. Lütjeharms,
Bloemfontein; H. Lundegårdh, Uppsala; E. Melin, Uppsala; M. Mes, Pretoria;
M. Ødelien, Ås; E. J. Petersen, Copenhagen; C. S. Piper, Adelaide; H. G. Thornton,
Harpندن, Herts.; S. Tovborg Jensen, Copenhagen; A. I. Virtanen, Helsinki; S. A.
Waksman, New Brunswick; F. W. Went, Pasadena; Joh. Westerdijk, Baarn;
J. G. Wood, Adelaide.

CONTENTS: TABLE DES MATIÈRES: INHALT:

W. R. Schell and J. V. Jordan, Anion-exchange studies of pure clays . . .	303
E. G. Mulder, K. Bakema and W. L. van Veen, Molybdenum in symbiotic nitrogen fixation and in nitrate assimilation	319
E. G. Mulder, R. Boxma, and W. L. van Veen, The effect of molybdenum and nitrogen deficiencies on nitrate reduction in plant tissues	335
✓ U. S. Moser, W. H. Sutherland, and C. A. Black, Evaluation of laboratory indexes of absorption of soil phosphorus by plants: I.	356
E. S. Twyman, The effect of iron supply on the yield and composition of leaves of tomato plants.	375
J. D. Ovington and H. A. I. Madgwick, The growth and composition of natural stands of birch: II. The uptake of mineral nutrients	389

Printed in the Netherlands

ANION-EXCHANGE STUDIES OF PURE CLAYS *

by W. R. SCHELL and J. V. JORDAN **

Anion-exchange reactions in clays have been recognized for some time but little is known of the factors involved in these reactions. There are varying theories based on different interpretations of data. Some authors state that anions such as phosphate, arsenate and borate, which are about the same size as the silicon tetrahedron, may be absorbed by replacement of the silica and by lattice extension. Schofield^{20 21} suggests that clay minerals may have anion-exchange sites on basal plane surfaces. Such active anion-exchange positions are due to unbalanced charges within the lattice.

Anion-exchange studies involve difficulties in the analysis and extraction of the anions. In these studies, these inherent problems were limited through the use of a special clay-preparation technique and by a unique extraction procedure utilizing electrodialysis. This approach to the exchange phenomenon may help establish some additional properties of clay systems.

Correlation studies were made on the natural clays comparing the surface area, both external and internal, with exchangeable anions. This aided in determining some of the structural characteristics of clay-water systems.

PURPOSE OF INVESTIGATION

Anion-exchange reactions in soils and clays are important in many fields of study. In soil chemistry these reactions may be a key to material balance in plant nutrition. The basic data on anion

* Published with the approval of the Director, Idaho Agricultural Experiment Station as Research Paper No. 423.

** Research Fellow and Associate Agricultural Chemist, respectively.

exchange is quite meager. This report attempts to establish an anion-exchange phenomenon and describe the methods of investigation used to determine certain anion-exchange reactions in clays.

LITERATURE REVIEW

Gazzari⁹ first noted exchange in 1819 when he observed that clay decolorized liquid manure and retained soluble substances. Way²⁴ in 1850 verified these observations. Mattson¹⁶ reported that more ammonium ion was absorbed when used with phosphate than when used alone at the same pH. Dean and Rubins^{4 5} found that soils showed a definite anion-exchange capacity, but the different species of anions were not adsorbed by soils to the same degree. Stout²³ found that finely ground kaolinite and halloysite took up many times their basic exchange capacity of phosphate, while bentonite took up phosphate to the extent of only one tenth its basic exchange capacity. Stout reasoned that the difference between the anion- and cation-exchange capacities is due to the availability of hydroxyl ions on the surface of the clay minerals. Black¹ concurred, reporting that phosphate ions replaced hydroxyl groups on the lattice layers inside the kaolinite crystal as well as those exposed on the outer surface. Kelly and Midgley¹⁴ noted a definite increase in pH of the clay-water suspension when phosphate, ammonium fluoride and sodium silicate were added. Dickman and Bray⁷ concluded that stoichiometric replacement of hydroxyl groups by fluoride ion takes place on kaolin.

Sieling²² found that anion exchange of kaolin was increased by ball milling or by heating with alkali. McGeorge¹⁷ utilized an electrodialysis cell to determine phosphate availability in soils. He found amperage control to be important for reproducible results. Bradfield³ is credited for the apparatus used by most investigators for electrodiffusion studies of cations and anions.

EXPERIMENTAL

Electrodialysis apparatus

The apparatus used for the electrodialysis of clays was a cell having three chambers like the Bradfield cell³, but differing in internal arrangement and volume. The Schell unit²⁰, shown in Figure 1, was designed to offset certain disadvantages inherent in the commonly used Bradfield cell. The apparatus comprised a 500-ml, double-neck distilling flask. Immersed in this large chamber was a cellulose dialyzer tube which carried the clay suspension to be electrodialyzed. Immersed in this tube was a smaller cellulose dialyzer tube which contained the cathode. Having this membrane around the cathode prevented encrustation of the electrode by the colloid and minimized any basic reaction from exchanging a greater amount of clay anions than would normally occur. The anode was suspended in the flask outside the

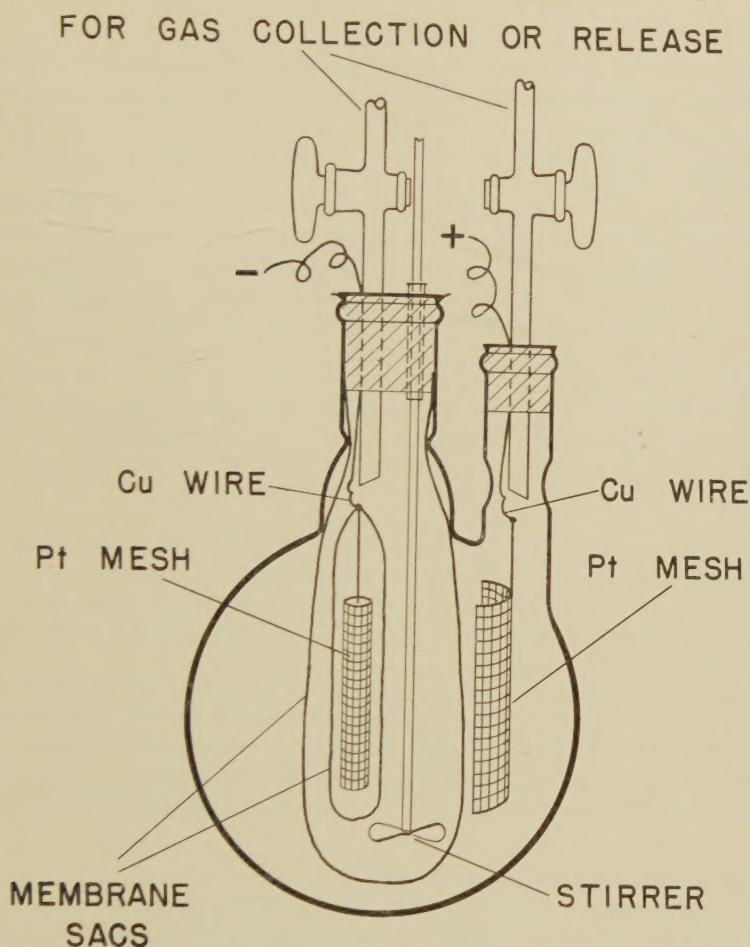


Fig. 1. Schell cell for electrodialysis of clay suspensions.

larger sac. The stirring device in the larger sac maintained the colloid in a homogeneous suspension during the electrodialysis. Whilst platinum mesh electrodes were used in this study other cheaper materials might be used. The entire unit was suspended in a controlled-temperature water bath. A Reco * transformer furnished power for the operation. Duplicate determinations were easily run by connecting two units in series.

In order to minimize resistance between the electrodes the large chamber was filled with 0.05 N H_3BO_3 ¹⁸. Samples were withdrawn from the chambers by lifting the stoppers sufficiently to permit insertion of pipettes.

* Reco transformer, Model E-800-2, capacity 750 volts, 200 milliamp.; Research Equipment Corp., Oakland, Calif.

Preparation of clays

The following clays were used in this study: Kaolinite, pyrophyllite, halloysite, and bentonite. The clays, obtained from various processors, were used in the "purified" state as obtained with a particle size averaging less than one micron. They did not contain appreciable amounts of the anions investigated to influence the data *.

The preparation of the clays was made using *N*/1 ammonium sulfate, *N*/1 ammonium phosphate, and *N*/1 ammonium chloride solutions. In some of the experiments microcurie levels of sulfur-35, phosphorus-32, and chlorine-36 were used as tracers. Five-gram portions of the clay were suspended in 45 ml H₂O. Five ml of the respective salt solution were then added and the contents equilibrated by agitation for a given time. Twenty-four hours was found to be enough. After equilibrium, the clays were removed from the salt solution by centrifugation and washed free of excess anion with alcohol until the washings gave no test for the anion. This was completed by washing 4 times with 20 ml ethanol. Following drying in a vacuum oven at 50°C, 4.50-gram portions of the prepared NH₄-clay-X (X = PO₄, SO₄, or Cl) were weighed out for electrodialysis. During electrodialysis the voltage was maintained at 200 volts for kaolin and pyrophyllite, and 150 volts for halloysite. Milliampere readings were taken at time intervals.

Relative specific activities of the tagged equilibrated clays were determined before and after electrodialysis by weighing 60.0 mg of the dried processed clay into planchets.

Bentonite was prepared by passing a 2 per cent Wyoming bentonite through a prepared NH₄-resin and SO₄-resin, Dowex 50 and Dowex 2, respectively. Fifty ml of the prepared bentonite suspension were electrodialyzed.

Surface-area measurements were made on the clay samples following their physical preparation and prior to any chemical equilibration procedures.

Physical and chemical determinations

The phosphorus exchanged from the clays was determined by the method of Dickman and Bray⁶ using an Evelyn photoelectric colorimeter. Sulfate exchanged from the clays was determined using the method of Kolthoff and Sandell¹⁵ on the extract solution from the anode compartment.

The radiotracer measurements were made using Q-gas in the RCI preflush windowless flow counter and a Tracerlab autoscaler.

Surface-area determinations were run using the ethylene glycol retention method as outlined by Bower and Oschwend² and Dyal and Hendricks⁸.

The electrokinetic measurements were made by reading the variance in milliamps during time increments while maintaining the voltage constant.

* The clays were obtained through courtesy of various manufacturers. Whittaker, Clark and Daniels Co. supplied kaolinite; J. M. Huber Corp. supplied halloysite, Grade 47, and Bentonite, Grade 50; Baroid Sales Division of National Lead Co. supplied Wyoming bentonite; Ward's Natural Science Establishment, Inc., supplied kaolinite, halloysite and pyrophyllite.

RESULTS AND DISCUSSION

The electrodialysis separation technique can be observed as a method of extraction only. The clay structure will be broken up by excessive current density, and upon electrodialysis additional valence sites could be formed. This would make a great difference in the exchange capacity. Since there were no appreciable anions present originally in the purified clays, the effect of cleavage of the crystalline structure and exchanging a greater amount of anions is eliminated. A technique for extraction by fluoride was tried early in the work but was found inappropriate due to difficulties in analysis and reactivity of the fluoride. The clays were washed free of adsorbed anions by the ethanol. Ethanol was used as a wash solution to minimize hydrolytic effect on the anion-exchanged clays.

The voltage was maintained constant at 200 volts for kaolinite and pyrophyllite to attain maximum current flow and efficiency of separation. It was necessary to lower the voltage to 150 volts with the halloysite to maintain the milliamps reading within the limits of the RECO transformer, the maximum output being 200 milliamps. This is illustrated in Figures 2, 3, and 4. These figures are not quantitative but do illustrate the difference in electrical nature of the clays which have adsorbed anions. In all cases the clays are ammonium saturated. The difference in migration is due to the anion present in their respective systems.

The electrokinetic curves for bentonite are not included. Due to its very high charge and ease of migration the voltage had to be kept below 25 volts to maintain the milliamp reading within the limits of the transformer. Bentonite exhibited a high anion-exchange capacity for both phosphate and sulfate. These anions would influence the current migration in conjunction with the large inherent charge on the bentonite micelle.

With the large excess of ammonium salts added, the foreign ions which may have been on the clays originally can be considered to have little or no effect on the data.

The surface area of clays is an important factor in anion-exchange capacity. This is evident in Table I where exchangeable sulfate varies from 52.67 milliequivalents per 100 grams bentonite down to 2.01 me/100 g pyrophyllite. Similarly, exchangeable phosphate

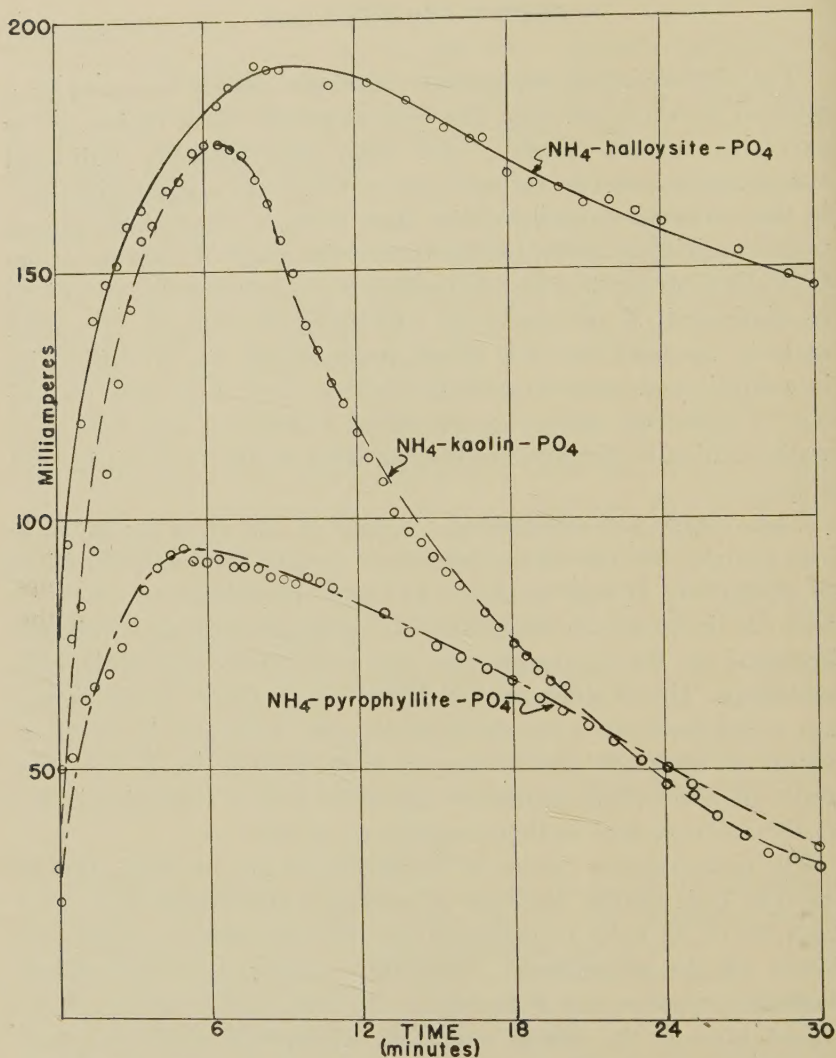


Fig. 2. Conductivity curves for NH_4 -kaolin- PO_4 , NH_4 -halloysite- PO_4 and NH_4 -pyrophyllite- PO_4 .

ranges from 50.20 me down to 5.55 me for the same clays. The exchangeable anions on halloysite and kaolin were intermediate in accordance with their surface-structural characteristics.

The large internal surface of bentonite may be attributed to its expanding lattice. On the other hand, the relatively larger internal

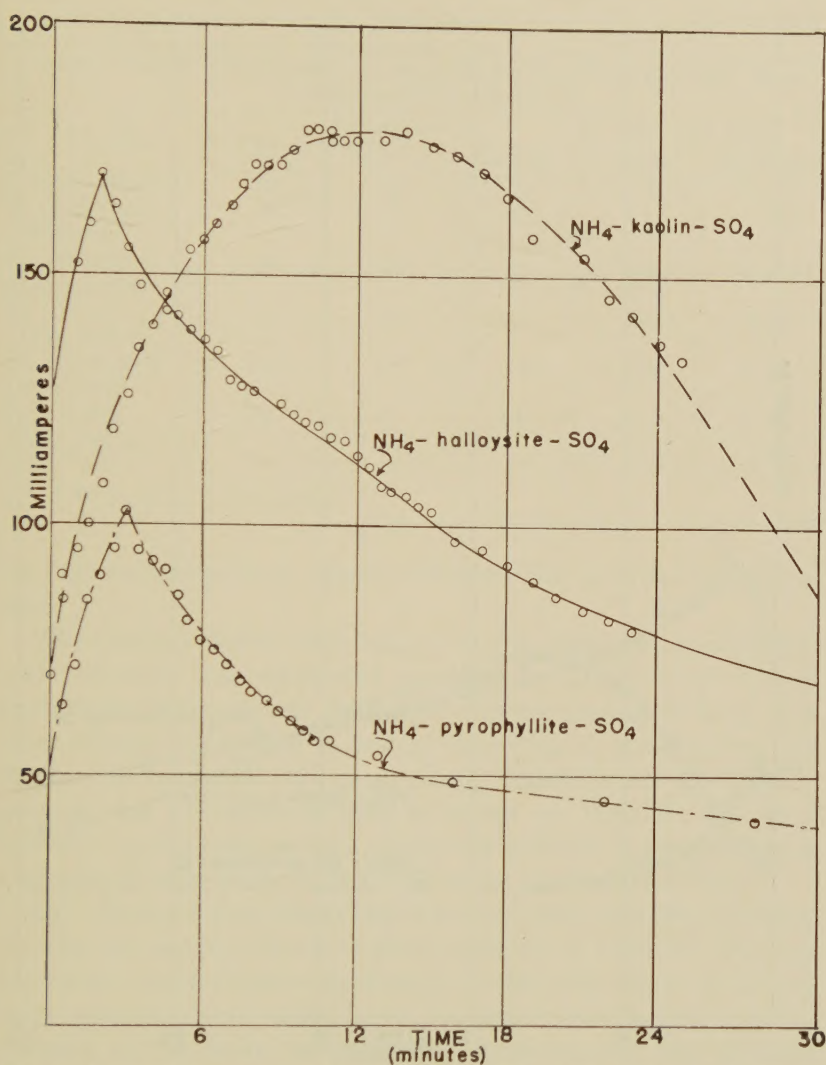


Fig. 3. Conductivity curves for NH_4 -kaolin- SO_4 , NH_4 -halloysite- SO_4 and NH_4 -pyrophyllite- SO_4 .

area of halloysite may be due to the greater water of hydration coordinated in its crystal. There is also the possibility that since halloysite crystals are tubular, the micelles rupture exposing a larger internal surface area. The kaolin crystal consists of only two interfaces and does not have an expanding lattice or water of

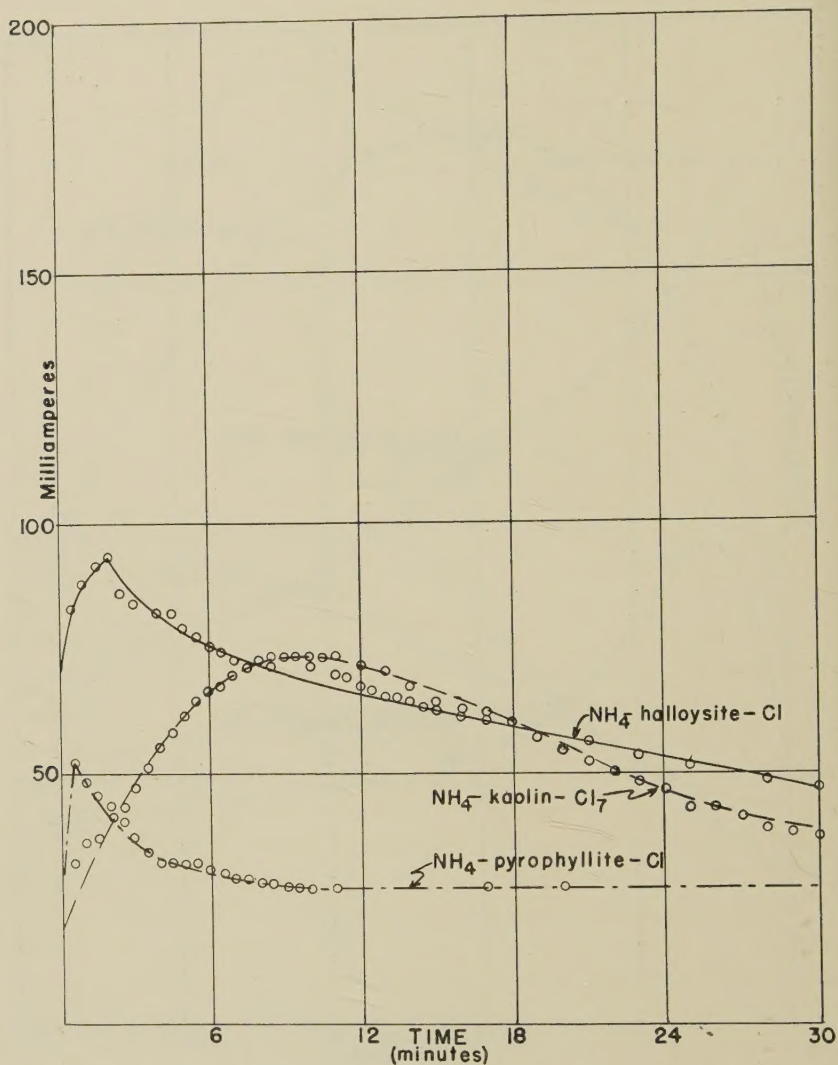


Fig. 4. Conductivity curves for NH₄-kaolin-Cl₇, NH₄-halloysite-Cl and NH₄-pyrophyllite-Cl.

hydration. Pyrophyllite has essentially the same crystalline structure as bentonite but the crystal does not expand. The external surface area comprises almost entirely the total surface area. The data indicate a definite relationship between surface area and anion-exchange capacity, giving credence to the hypothe-

TABLE I

Effect of surface area (sq.m/g) on exchangeable sulfate and phosphate (me/100 g) as determined by electrodialysis					
Clay type	Surface area			Exchange anions	
	Total *	External	Internal	Sulfate	Phosphate
Kaolin (Ward's)	47	19	28	5.42	7.97
Kaolin (Huber)	24	11	13	4.01	9.22
Halloysite (Filtrol)	200	136	64	13.84	25.51
Pyrophyllite (Ward's)	15	12	3	2.01	5.55
Pyrophyllite (Whittaker)	5	6	0	1.55	6.13
Bentonite (Filtrol)	787	307	480	52.67	50.20
L.S.D. (0.01)				2.03	1.83

* Averages of 2 determinations.

sis that anion-exchange capacity is essentially a surface phenomenon.

When surface-active clays such as kaolin, halloysite, pyrophyllite and bentonite are suspended in solutions of 0.1N ammonium phosphate or ammonium sulfate for varying periods of time, each type adsorbs phosphate or sulfate according to its characteristic internal and external surface structure. This effect on the clays of varying the equilibration time is shown in Table II. The data indicate that the main surface adsorption by these clays of phosphate and sulfate takes place in 24 hours or less equilibration time.

The effects of electrodialyzing $\text{NH}_4\text{-clay-PO}_4$ and $\text{NH}_4\text{-clay-SO}_4$ samples for varying periods of time are shown in Table III. The two anions are not necessarily exchanged to the same degree by all the clays studied. In all cases, more phosphate than sulfate was exchanged by the clays, therefore indicating that the clay surfaces were occupied by more phosphate anions. The phosphate exchanged from bentonite increased slightly as time of electrodialysis was extended beyond 5 hours. This may have been due to phosphate substitution in the bentonite crystal during equilibration and these bonds rupturing upon continued electrodialysis. Phosphate exchanged from halloysite increased with time of electrodialysis. This may be attributed to both the tubular nature of the halloysite crystal and the breaking up of the crystalline structure by electro-

TABLE II

Effect of equilibration time on the amount of phosphate and sulfate (me/100 g) exchanged by various clays			
Clay type	Equilibration time, hours	Anion exchanged	
		Phosphate	Sulfate
Kaolin (Huber)	24	9.83	4.04
" "	48	9.72	3.49
" "	96	9.88	3.88
" (Ward's)	24	8.26	5.42
Halloysite (Filtrol)	24	22.70	13.00
" "	48	24.80	13.12
" "	96	24.30	14.56
" (Ward's)	24	20.73	10.31
Pyrophyllite (Whittaker)	24	4.99	1.64
" "	48	5.28	1.57
" "	96	5.62	1.68
" (Ward's)	24	5.55	2.01
Bentonite (Filtrol)	24	51.48	53.20
" "	48	56.60	54.70
" "	96	56.60	—

dialysis. Phosphate bonded on the inner tubular surfaces was less easily extracted.

Referring to the sulfate exchanged from the five clay types listed in Table III, it is evident that extending the time of electro dialysis did not increase the sulfate exchanged off the clay surfaces. Electro dialyzing the equilibrated clays for three to five hours removed rather constant amounts of sulfate. Phosphate and sulfate remaining on the clay surfaces after the aforementioned periods of electro dialysis are dealt with in discussion pertaining to Table IV. Chloride exchange was attempted on the clays but the concentration of this anion extracted by electro dialysis was so low that chemical analysis was impractical, although radioanalysis was successful.

Data illustrating the adsorption and exchange of phosphate, sulfate and chloride on clays using radiotracers are found in Table IV. Here the clays were equilibrated with the respective $N/10$ ammonium salt solutions carrying tracer levels of P^{32} , S^{35} and Cl^{36} . The specific activities of these equilibrated clays were determined before and after electro dialysis. Results show that

TABLE III

Effect of time of electrodialysis (hours) on sulfate and phosphate exchanged from clays (me/100 g).				
Clay type	Time for electro-dialysis	Phosphate exchanged	Time for electro-dialysis	Sulfate exchanged
Kaolin (Huber)	0.5	4.00	4.5	3.16
" "	1	8.22	5.75	3.59
" "	1.5	8.77	7.5	3.64
" "	2.5	9.22	—	—
" "	3.0	9.22	—	—
" "	3.5	9.22	—	—
" "	4.5	9.22	—	—
" "	4.0	9.22	—	—
Halloysite (Filtrol)	2	12.59	3	13.88
" "	4.5	12.62	4	14.22
" "	6.5	19.40	5	14.45
" "	8.5	20.37	8.25	14.67
" "	12.5	21.33	—	—
" "	23.5	28.32	—	—
Pyrophyllite (Whittaker)	2	5.04	—	—
" "	3	4.94	3	1.96
" "	4	6.10	—	—
" "	6	5.55	—	—
Bentonite (Filtrol)	2	33.37	3	55.14
" "	4	51.00	4	53.20
" "	5	56.55	4.5	51.97
" "	8.5	57.77	—	—
" "	11.25	60.15	—	—
" (Baroid)	—	—	3	54.17
" "	—	—	6	55.30

relatively more phosphate was retained by the clays after electrodialysis than were sulfate and chloride. The descending order of retention for phosphate by the clays was as follows: bentonite, halloysite, kaolinite and pyrophyllite. This is similar to the order of phosphate anion-exchange capacity.

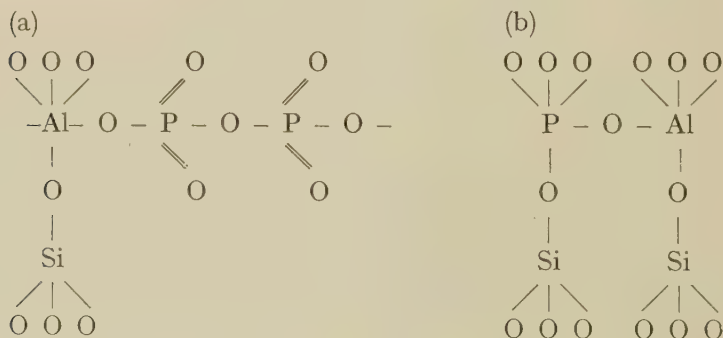
Apparently phosphate in the NH_4 -halloysite- PO_4 and NH_4 -bentonite- PO_4 forms is not in entirely ionizable or exchangeable states due to activity retention by the clays. Such a phenomenon may be explained by a lattice-extension theory mentioned by Hendricks¹¹ wherein phosphate coordinates in the sphere of the

TABLE IV

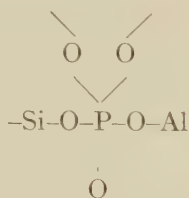
Specific activity (counts per minute per gram) of clays tagged with P^{32} , S^{35} and Cl^{36} before and after electro dialysis extraction						
Clay type	Specific activity * (c.p.m./unit wt. of tagged clay)					
	Clay- $P^{32}O_4$		Clay- $S^{35}O_4$		Clay- Cl^{36}	
	Before dialysis	After dialysis	Before dialysis	After dialysis	Before dialysis	After dialysis
Kaolin (Ward)	1,403	0	11,208	0	18,610	0
Kaolin (Huber)	1,543	0				
Halloysite (Ward)	4,935	2,616	11,721	2,520	1,701	0
Halloysite (Filtrol)	5,677	2,184				
Pyrophyllite (Ward)	755	0	675	0	523	0
Pyrophyllite (Whittaker)	1,127	0				
Bentonite (Filtrol)	4,859	2,976	37,316	1,502	31,316	469

* Averages of duplicate electro dialyses extractions.

aluminate silicate crystal both by replacement and by extension adsorption. These may be illustrated as follows:



Phosphate adsorption on clays may involve both mechanisms. When the phosphate is polymerized in (a), the bonding weakens along the phosphate extension. A semi-polar solvent such as ethanol was used to wash out all weakly bound phosphate, and the phosphate remaining represented the exchanged and lattice-substituted phosphate. In example (b) the phosphate is coordinated within the crystal and is not readily ionized except under extreme conditions, such as electro dialysis, which tends to break the



bonds. Aluminate, silicate and phosphate are quite similar in their coordination structure so that the possibility of lattice substitution and lattice extension by phosphate seems reasonable. Grim¹⁰ mentions that the actual formula for montmorillonite always differs from the theoretical formula because of substitutions within the aluminate and possible silicate lattices in tetrahedral coordinated positions. Iler¹² states that the -Al-OH bond breaks enabling phosphate and pyrophosphate to substitute at these sites.

Electrodialysis removed practically all phosphate from equilibrated kaolin and pyrophyllite. Sulfate, like phosphate, was adsorbed in relatively large amounts by bentonite and probably in the same manner by lattice substitution. Data in Table III indicate the sulfate was less tightly held than phosphate on the clay micelles.

Since the radioactive species used as shown in Table V have different decay patterns and emit beta particles having quite different energy levels, the activities of the $\text{NH}_4\text{-clay-PO}_4$, $\text{NH}_4\text{-clay-SO}_4$ and $\text{NH}_4\text{-clay-Cl}$ are not comparable one with the other. However, the activities of each anion exchanged on the various clays are comparable; for example, 4859 counts per minute per gram of bentonite versus 755 counts per minute per gram for pyrophyllite.

Although zero activity values are referred to in certain cases, this does not imply that electrodialysis removed all the anions from the equilibrated clays. Rather, the inference is that the specific activities of these particular samples after electrodialysis were so low as not to be significantly different than zero.

Figures 2, 3 and 4 display graphically the difference in ion migration and electrical properties of the various clay types. In all cases the clays were saturated with ammonium ion so that the ion migrations observed depended on the electrically charged

characteristics of the individual clay, specifically the migration of phosphate, sulfate and chloride ions. The migration of sulfate and chloride ions from both halloysite and pyrophyllite approached a maxima in less than three minutes, then decreased rapidly with time. The peaks reached by the NH_4 -kaolin-Cl, NH_4 -halloysite-Cl and NH_4 -pyrophyllite-Cl were much lower than those for the sulfate and phosphate migration patterns. This was probably due to three factors: (1) the monovalent chloride ion carried a relatively smaller per cent of the current than the sulfate and phosphate ions; (2) a relatively smaller amount of chloride was exchanged on the clays; and (3) the chloride ion was more readily exchanged than sulfate or phosphate. In Figure 2, the pattern of the conductivity curve for NH_4 -halloysite- PO_4 can be explained on the basis of data in Tables III and IV. Halloysite tended to hold phosphate more tightly than the other two anions, both externally and within the tubular surfaces. The migration of these ions was necessarily slower and the phosphate anion carried the highest charge. These factors maintained the milliamperage at a high level for a longer time until all the anions were exchanged through the semi-permeable membrane sac in the electrodialysis cell. Apparently the exchangeable phosphate was electrodialyzed off kaolin and pyrophyllite more readily, as illustrated by the conductivity curves in Figure 2.

SUMMARY AND CONCLUSIONS

Anion-exchange studies on pure kaolin, pyrophyllite, halloysite and bentonite indicate there exists a definite relationship between anion-exchange capacity and the surface chemico-physical properties of the clays. From the data in this study the following conclusions may be drawn:

1. The Schell cell for electrodialysis gave reproducible results for the anions, phosphate, sulfate and chloride, and is convenient to operate.
2. A relationship exists between the total surface area of bentonite, halloysite, kaolinite and pyrophyllite and the amount of phosphate or sulfate ions these clays adsorbed or exchanged.
3. Bentonite, having the largest total surface area, also had the largest internal surface area. This clay exhibited the greatest anion-exchange capacity, whereas pyrophyllite, with a small total surface area, exhibited a relatively low anion-exchange capacity.
4. Kaolin, halloysite and pyrophyllite adsorbed more phosphate than sulfate, but bentonite adsorbed equivalent amounts of both these anions.

5. Under the conditions of equilibration, the four clay types adsorbed close to maximum amount of phosphate and sulfate in 24 hours or less.

6. Activity of retention or the bonding of the anions on the different clays is indicated by the length of time required for the electrodialysis process to remove the anions.

7. The clays adsorbed only traces of chloride according to chemical analysis of the electrodialysate. Radioanalysis indicated that all clays did actually adsorb chloride.

8. Prolonged electrodialyses of the tagged clays suggested that the clays, particularly halloysite and bentonite, adsorbed the anions partly by substitution in the lattice for aluminum or silicon. Readily exchangeable anions were explained as being adsorbed on the exchangeable sites and/or by lattice extension.

Received June 24, 1958

REFERENCES

- 1 Black, C. A., The penetration of phosphate kaolinite crystal. *Soil Sci. Soc. Am. Proc.* **6**, 157-161 (1941).
- 2 Bower, C. A., and Oschwend, F. B., Ethylene glycol retention by soils as a measure of surface area and interlayer swelling. *Soil Sci. Soc. Am. Proc.* **16**, 342-345 (1952).
- 3 Bradfield, R., The use of electrodialysis in physico-chemical investigations of soils. *Proc. First Intern. Congr. Soil Sci.*, 264-278, June (1927).
- 4 Dean, L. A., and Rubins, E. J., Anion exchange in soils: I. Exchangeable phosphorus and the anion exchange capacity. *Soil Sci.* **63**, 377-387 (1947).
- 5 Dean, L. A., and Rubins, E. J., Anion exchange in soils: III. Application to problems of soil fertility. *Soil Sci.* **63**, 399-406 (1947).
- 6 Dickman, S. R., and Bray, R. H., Colorimetric determination of phosphate. *Ind. Eng. Chem., Anal. Ed.* **12**, 665-668 (1940).
- 7 Dickman, S. R., and Bray, R. H., Replacement of adsorbed phosphate from kaolinite by fluoride. *Soil Sci.* **52**, 263-273 (1941).
- 8 Dyal, R. S., and Hendricks, S. B., Total surface of clays in polar liquids as a characteristic index. *Soil Sci.* **69**, 421-432 (1950).
- 9 Gazzari, J., Zurückhaltung auflösbarer Substanzen durch Lehm von Dünge-Jauche. Cf. Sistini, P., *Landw. Vers. Sta.* **16**, 409-411 (1873).
- 10 Grim, R. E., *Clay Mineralogy*. McGraw-Hill Co., N.Y. 384 pp. (1953).
- 11 Hendricks, S. B., Lattice structure of clay minerals and some properties of clays. *J. Geol.* **50**, 267-290 (1942).
- 12 Iler, R. K., *The Colloidal Chemistry of Silica and Silicates*. Cornell Univ. Press, Ithaca, N.Y. 324 pp. (1955).
- 13 Kelley, J. B., and Midgley, A. R., Phosphate fixation - an exchange of phosphate and hydroxyl ions. *Soil Sci.* **55**, 167-176 (1943).
- 14 Kolthoff, I. M., and Sandell, E. B., *Textbook of Quantitative Organic Analyses*. The Macmillan Co., N.Y. 759 pp. (1952).
- 15 Mattson, S., and Pugh, A. J., The laws of soil colloid behaviour: VII. Amphoteric behavior. *Soil Sci.* **32**, 299-313 (1931).
- 16 McGeorge, W. T., Electrodialysis as a Measure of Phosphate Availability in Soils and the Relation of Soil Reaction and Ionization of Phosphates to Phosphate Assimilation. *Univ. Ariz. Tech. Bull. No.* **38** (1932).

- 17 Purvis, E. R., and Hanna, W. S., Rapid electrodialysis of soils in dilute boric acid solution. *Soil Sci.* **67**, 47-52 (1949).
- 18 Ravikovitch, S., Anion exchange. 1. Adsorption of the phosphoric acid ions by soils. *Soil Sci.* **38**, 219-239 (1934).
- 19 Schell, W. R., and Jordan, J. V., Apparatus for electrodialysis. *Chemist-Analyst* **45**, 56 (1956).
- 20 Schofield, R. K., Clay mineral structures and their physical significance. *Trans. Ceram. Soc. (Eng.)* **39**, 147-158 (1940).
- 21 Schofield, R. K., Calculation of surface area of clays from measurements of negative adsorption. *Trans. Ceram. Soc. (Eng.)* **48**, 207-213 (1949).
- 22 Sieling, D. H., Role of kaolin in anion sorption and exchange. *Soil Sci. Soc. Am. Proc.* **11**, 161-170 (1946).
- 23 Stout, P. R., Alterations in the crystal structures of clay minerals as a result of phosphate fixation. *Soil Sci. Soc. Am. Proc.* **4**, 177-182 (1939).
- 24 Way, J. T., On the power of soils to absorb manure. *J. Royal Agr. Soc. Engl.* **11**, 313-379 (1850).

MOLYBDENUM IN SYMBIOTIC NITROGEN FIXATION AND IN NITRATE ASSIMILATION *

by E. G. MULDER, K. BAKEMA **, and W. L. VAN VEEN

Laboratory of Microbiology, Agricultural University,
Wageningen, the Netherlands.

INTRODUCTION

The essentiality of molybdenum as a catalyst in nitrogen fixation by free-living micro-organisms has been demonstrated by several workers ^{7 9 10 13 16 22} following the discovery by Bortels ⁸ in 1930 that this element is a micronutrient for *Azotobacter chroococcum*. That molybdenum is also of major importance in symbiotic nitrogen fixation was shown by the senior author in culture-solution experiments with pea plants ¹⁶. In the absence of added molybdenum the nodules were somewhat smaller than those of normal plants. They had not the normal pink shade, but were of a pale yellow-brown colour. Their nitrogen-fixing capacity was very poor as was concluded from nitrogen analyses and from the light-green colour of the molybdenum-deficient plants as contrasted to the dark green of molybdenum-treated plants. These experiments confirmed earlier findings of Anderson ^{3 4} that the addition of small amounts of molybdenum to certain Australian soils had a remarkable effect on nitrogen fixation by clover and lucerne plants.

The essentiality of molybdenum for nitrate assimilation was demonstrated by experiments with *Aspergillus niger* ^{16 21 25 26}, *Neurospora crassa* ²¹, algae ^{5 12} and higher plants ^{16 17}. Denitrifying bacteria require also molybdenum when growing under anaerobic conditions in the presence of nitrate ¹⁶.

* The major part of this work was carried out by the authors at the Institute for Soil Fertility, Groningen.

** Institute for Soil Fertility, Groningen.

The mechanisms of nitrogen fixation, symbiotic as well as non-symbiotic, have not so far been cleared up. As a consequence the precise function of molybdenum in these processes is still obscure. Shug *et al.*²⁴ have found that hydrogenase requires molybdenum for the reduction of cytochrome c or other one-electron-accepting compounds but not for the reduction of two-electron acceptors like methylene blue. Hydrogenase is postulated by some authors to play a specific role in the reduction of N_2 to ammonia. This is concluded from the fact that nitrogen-fixing organisms contain relatively large amounts of hydrogenase, while factors which inhibit nitrogen fixation, as nitrate, also inhibit the formation of hydrogenase^{11 14 23}. It must be stated, however, that no hydrogenase has been detected in *Rhizobium* cells when grown in the laboratory or when taken directly from the nodule (Shug *et al.*²³), while mutants of *Azotobacter vinelandii* which are unable to fix nitrogen contain 50 per cent of the hydrogenase activity of the active nitrogen fixers¹. Both these observations are not consistent with the essentiality of hydrogenase in nitrogen fixation. In experiments with *Azotobacter* and *Clostridium*, Nicholas²⁰ found that molybdenum deficiency decreased nitrogen fixation but it did not markedly reduce hydrogenase activity.

That nitrogen fixation is a reductive process was shown by investigations with *Clostridium pasteurianum*³⁰, *Azotobacter vinelandii*^{1 19} and with nodules from soybean plants³¹. The cultures were exposed for a short period of time to an N^{15}_2 -containing gas mixture and subsequently the distribution of N^{15} among various N-compounds of the cells and the culture solution was estimated. Evidence was obtained that the first detectable compound after fixation is ammonia from which subsequently glutamic acid is formed.

In order to secure information upon the sequence of compounds during the assimilation of N^{15}_2 , the cultures must be killed a short time after their exposure to N^{15}_2 . The nitrogenous compounds, preferably those not yet incorporated in cellular proteins, must be isolated and analysed for N^{15} . Since these fractions generally are present in small amounts, their isolation is extremely difficult.

The purpose of the present investigation was to study the mechanism of symbiotic nitrogen fixation as well as that of nitrate assimilation by using molybdenum-deficient plants to which small

amounts of sodium molybdate are added. Since nitrogen-fixation reactions have practically come to a standstill in molybdenum-deficient nodules, their content of non-protein nitrogenous compounds is very low. Addition of molybdate restores nitrogen fixation so that by estimating various nitrogenous fractions after appropriate periods of time, the sequence of reactions in nitrogen fixation may be studied. Isolation of these compounds as in the case of N^{15} -tests is superfluous.

In the present work a study was made of the synthesis of amino acids in the nodules of molybdenum-deficient lucerne and clover plants when small amounts of molybdate were added to their nutrient medium. In similar experiments the synthesis of amino acids from accumulated nitrate was investigated in molybdenum-deficient cauliflower, spinach, and tomato plants after the addition of molybdate.

EXPERIMENTAL

Cultivation of plants

Lucerne, red clover, cauliflower, spinach, and tomato plants were grown in Neubauer jars containing 0.5 or 1 kg of molybdenum-deficient soil. In the case of leguminous plants and spinach approximately 20 plants were cultivated in small jars and 30 in large jars; with cauliflower and tomato the number of plants per pot was about one-half and one-third, respectively, of that with spinach. The basic dressing given to these pots consisted of 0.6 g K_2HPO_4 and 0.1 g $MgSO_4 \cdot 7H_2O$ per kg of soil. Some of the pots received 5 mg $Na_2MoO_4 \cdot 2H_2O$ per kg of soil. In order to eliminate a possible sodium effect of the molybdate 3.0 mg $Na_2CO_3 \cdot 10H_2O$ was added to the control pots. In the case of leguminous plants the soil had to be inoculated with a suspension of the appropriate *Rhizobium* species.

When the molybdenum-deficient lucerne and clover plants had been cultivated for approximately 8 to 9 weeks, 5 mg $Na_2MoO_4 \cdot 2H_2O$ in 10 ml glass-distilled water was added per kg of soil. This solution was washed into the soil with a further 20 ml H_2O . Control pots received distilled water only.

The plants were incubated in a greenhouse at 20 to 25°C under natural light conditions and after periods of time varying from 5 hours to 10 days the nodules from 5 pots were collected after careful washing of the roots on a sieve.

The cauliflower, spinach, and tomato plants which were used for studying the effect of molybdenum on nitrate assimilation received in addition to the basic dressing mentioned above 1.5 g KNO_3 per pot containing 1 kg of soil. In order to produce plants of different deficiency levels one series of pots was left without molybdenum, whilst a second series was dressed with 50 μg , and a third with 2.5 mg $Na_2MoO_4 \cdot 2H_2O$ per pot. After 4 to 5 weeks, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$ dissolved in 10 ml H_2O

was added to some of the pots with molybdenum-deficient plants and washed into the soil as described above. After different periods of time, plants were harvested and used for various tests.

Analytical methods

Preparation of the samples. In the case of nodules, 1 to 4 g of fresh tissue was ground in a chilled mortar and treated with 200 ml of 80% ethanol to precipitate the protein. The latter was separated by filtration and the filtrate concentrated at 35°C to approximately 1 ml. The concentrate was made up to 5 or 6 ml with distilled water and the free amino acids estimated by paper chromatography.

In the case of cauliflower, spinach, and tomato, average samples of all the laminae – excepting those which showed symptoms of necrosis – were taken from a number of pots. Ten to twenty grams of cut leaf tissue were ground in a porcelain mortar and transferred with 200 ml H₂O to Erlenmeyer flasks.

These were heated in a water-bath at 90°C for 5 minutes. After rapid cooling the samples were kept for a few days at 3°C. The precipitated protein was separated by filtration on a Büchner funnel and washed three times with distilled water. The filtrates, which contained the free amino acids, were concentrated *in vacuo* at 35°C to 10 ml.

Estimation of amino acids by quantitative paper chromatography. The method used, which is fully described elsewhere¹⁸ consists of a two-directional descending procedure with phenol-water and collidine-lutidine-water as the moving phases. Development of the chromatograms took place in a special cabinet in which they were heated under ethanol-saturated anaerobic conditions so that an optimal colour intensity was obtained^{18 27 28}. After the papers had been dried, the coloured spots were cut out, extracted with 10 ml 50% ethanol and the intensity of the colour measured in a spectrophotometer.

Protein and soluble non-protein and nitrate nitrogen were determined in the samples used for amino-acid determination or in separate samples of 1 to 10 g of ground fresh plant tissue. In the latter case the macerates were transferred with distilled H₂O to 100-ml Erlenmeyer flasks, heated for 15 minutes at 90°C and after cooling treated with 12.5 ml of a 20% solution of trichloro-acetic acid. Subsequently they were filtered on a Büchner funnel, and the precipitates washed several times with a 1% solution of trichloro-acetic acid. Residues as well as filtrates were analysed for nitrogen according to the Kjeldahl-Lauro method. Nitrate was removed from the filtrates by diluting 10 to 20 ml with 50 ml of distilled H₂O and heating the mixture with 5 ml of concentrated H₂SO₄ and 2 g of selenium catalyst. During the concentration of this solution, nitrate was quantitatively decomposed and removed¹⁵. Nitrate was estimated in a separate aliquot according to the xylenol method².

RESULTS

 1. *Synthesis of amino acids in nodules of molybdenum-deficient lucerne and clover plants after addition of molybdate*

a) First experiment with lucerne plants (14 to 28 September 1954). Nodules were collected 3, 6, 8, 10, and 14 days after application of 5 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ to lucerne plants with pronounced symptoms of molybdenum deficiency. Control plants, which had received water only, were harvested at 0 and 14 days – plants from soils treated with molybdenum before sowing the lucerne seeds, at 14 days. At each harvest time the nodules of three replicate pots were collected.

Three days after the addition of molybdate to molybdenum-deficient lucerne plants considerable increases in the α -alanine, glutamic acid, γ -aminobutyric acid, and asparagine contents were found (Table I). The protein content was also higher. At six days the

TABLE I

Effect of the addition of molybdate to molybdenum-deficient lucerne plants on the content of free amino acids in their root nodules (mg amino acids per g dry matter) *.								
Plants	Molybdenum-deficient							Normal
Treatment	No Mo added		5 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ added					(Mo before sowing only)
Days after treatment . .	0	14	3	6	8	10	14	14
<i>Amino compound **</i>								
Glycine	0.1	tr	0.2	0.3	tr	tr	tr	tr
α -Alanine	0.7	0.6	2.0	2.7	1.9	1.4	1.2	1.1
Serine	0.3	tr	0.4	0.7	0.3	0.7	tr	0.3
Valine	0.2	0.2	0.4	0.6	0.5	0.5	tr	0.3
Leucines	0.3	0.3	0.4	0.5	tr	0.7	tr	0.4
Proline	tr	tr	tr	tr	tr	tr	1.7	tr
Aspartic acid	0.3	tr	0.4	0.6	0.8	0.7	0.5	0.5
Glutamic acid	4.0	3.2	5.4	6.8	5.6	4.9	4.1	4.3
Arginine	tr	tr	tr	tr	0.6	tr	0.5	1.5
γ -Aminobutyric acid	0.6	0.3	1.3	1.9	1.4	1.1	2.0	1.4
Asparagine	18.4	14.1	22.4	54.3	48.5	63.8	64.7	49.2
Glutamine	0.5	tr	0.5	1.4	0.6	tr	tr	0.4
Soluble non-protein N †	9.5	9.1	10.5	19.8	23.5	19.5	22.6	21.3
Protein-N †	37.6	34.8	45.6	52.8	59.5		47.7	52.0

* Averages of quadruplicate values

** Cystine + cysteine, and threonine were found in traces. Methionine + methionine sulphoxide, tyrosine, and lysine were absent.

Phenylalanine, tryptophane, and histidine were not estimated.

† mg per g dry matter.

free amino acids were only slightly higher than at the previous harvest but by contrast asparagine had more than doubled. This rise of asparagine content continued until the tenth day after molybdenum treatment. α -Alanine and glutamic acid reached their maximal values six days after molybdenum treatment. Although the results of this experiment provide evidence that glutamic acid and perhaps α -alanine are formed first whereas asparagine may be considered as a secondary product, it is clear that more conclusive evidence might have been obtained if the nodules had been harvested at shorter time-intervals. Therefore in a second experiment with lucerne plants, the nodules were harvested at 5, 24, and 48 hours after molybdenum treatment.

b) Second experiment with lucerne plants (26 to 28 July 1955). The results of this experiment are recorded in Table II. The protein content as well as the soluble non-protein nitrogen and asparagine contents of the molybdenum-deficient nodules were considerably lower than in the first experiment. Five hours after

TABLE II

Effect of the addition of molybdate to molybdenum-deficient lucerne plants on the content of free amino acids in their root nodules (mg amino acid per g dry matter) *					
Plants	Molybdenum-deficient				Normal
Treatment	No Mo added	5 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ added			(Mo before sowing only)
Hours after treatment . . .	0	5	24	48	24
<i>Amino compound **</i>					
Glycine	tr	0.1	0.2	0.2	tr
α -Alanine	0.9	1.2	2.2	2.4	1.2
Serine	tr	0.3	0.4	0.5	tr
Valine	tr	tr	0.3	0.4	0.3
Leucines	tr	tr	0.5	0.7	0.3
Aspartic acid	tr	0.6	0.5	0.8	1.5
Glutamic acid	2.7	4.7	5.0	7.5	4.6
γ -Aminobutyric acid	0.5	1.2	1.4	2.0	1.1
Asparagine	10.4	9.2	10.7	17.1	55.4
Soluble non-protein-N †	3.2	4.4	5.8	8.5	22.9
Protein-N †	32.2	32.2	38.3	43.5	56.8

* Averages of quadruplicate values.

** Threonine, methionine + methionine sulphoxide, tyrosine, proline, arginine, and glutamine were found in traces only; cystine + cysteine, and lysine were absent; phenylalanine, tryptophane, and histidine were not estimated.

† mg per g dry matter.

application of molybdate to Mo-deficient plants, the glutamic-acid content had increased from 2.7 to 4.7 mg per g of dry nodule tissue. α -Alanine was also slightly higher; probably it is formed by transaminase activity from glutamic acid. The same may be the case with γ -aminobutyric acid which is a small but consistent constituent of the soluble non-protein fraction. Asparagine had not increased after 5 and 24 hours, and only slightly after 48 hours. This demonstrates clearly that asparagine does not belong to the primary reaction products in nitrogen fixation of lucerne plants.

In a subsequent experiment with white clover, which showed less pronounced symptoms of Mo-deficiency than the lucerne plants, harvests of nodules were made 28, 47, and 71 hours after addition of molybdate to molybdenum-deficient plants. As with the molybdenum-deficient lucerne plants, α -alanine, aspartic acid, glutamic acid, γ -aminobutyric acid, and particularly asparagine were the only amino compounds which occurred to any extent in the free state in the clover nodules. Twenty-eight hours after the addition of molybdenum to molybdenum-deficient plants an increase had occurred in α -alanine, γ -aminobutyric acid and asparagine; the glutamic acid content had hardly increased. After 47 hours, however, the glutamic-acid concentration had changed from 2.4 to 3.2, and the asparagine concentration from 7.4 to 28.7 mg per g of dry tissue.

2. *Synthesis of amino acids from nitrate in molybdenum-deficient cauliflower, spinach, and tomato plants after addition of molybdate*

a) *Experiment with cauliflower plants (7 to 9 July 1955).*

Three types of plants were used in this experiment, viz plants grown without added molybdenum, and plants dressed either with 50 μ g or with 2.5 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. Plants of the first type were small and showed severe symptoms of molybdenum deficiency, those of the second type showed moderate symptoms of Mo-deficiency whereas those receiving a dressing of 2.5 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ were well developed and had dark-green leaves.

Free amino acids in the leaves were determined at 18 and 42 hours after the addition of 2.5 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ to plants of the first two types. Control plants of all three types treated with water only were also analysed (see Table III). It will be seen from these data that 17 hours after the application of molybdate to molybdenum-deficient cauliflower plants the content of the following amino compounds of

TABLE III

Effect of the addition of molybdate to cauliflower plants with different molybdenum deficiencies on the content of free amino acids in their leaves (mg amino acid per g dry matter). *								
Amino compound **	Basic dressing only		Basic dressing					
			+ 50 μ g Na ₂ MoO ₄ . 2H ₂ O				+ 2.5 mg Na ₂ MoO ₄ . 2H ₂ O	
	17 h after addition of		18 h after addition of		42 h after addition of		18 h after addition of	
	H ₂ O	Mo	H ₂ O	Mo	H ₂ O	Mo	H ₂ O	
Glycine	0.5	3.6	0.3	0.9	1.2	3.1	0.2	
α -Alanine	1.0	2.2	0.7	1.4	1.0	1.3	1.2	
Serine	0.6	1.8	1.0	1.1	1.4	1.3	1.0	
Threonine†	1.0	1.1	0.4	0.4	0.6	0.7	0.4	
Aspartic acid	1.0	3.2	2.1	2.3	2.0	2.5	1.4	
Glutamic acid	3.1	5.0	4.1	4.1	5.1	5.7	3.2	
γ -Aminobutyric acid	0.4	0.4	0.9	1.5	0.9	1.0	1.9	
Glutamine	1.8	7.5	1.4	2.4	2.0	4.3	1.9	
Soluble non-protein-N ††	9.9	12.7	9.8	11.0	10.3	11.0	7.7	
Protein-N ††	32.0	34.1	38.4	41.6	36.8	40.2	41.1	
NO ₃ -N ††	29.8	20.1	12.6	11.3	7.3	7.1	1.5	

* Averages of quadruplicate values.

** Valine, methionine + methionine sulphoxide, leucines, proline, and lysine were found in traces only; cystine + cysteine, tyrosine, arginine, and asparagine were absent; phenylalanine, tryptophane, and histidine were not estimated. Close to glutamine a strongly coloured spot of an unknown amino compound was found.

† The spot of threonine on the chromatogram may have consisted partly of homoserine (cf Ref. 6²⁹).

†† mg per g of dry tissue.

the non-protein fraction had risen enormously: glycine, α -alanine, serine, aspartic acid, glutamic acid and glutamine. In the plants with slight deficiency symptoms a pronounced increase due to molybdenum treatment was found only with glycine and glutamine. The very high content of free glycine in these plants after addition of molybdenum is very remarkable, especially since the normal plants, which had received an adequate supply of molybdenum during their entire development, contained very little. The normal plants were also relatively low in glutamine and high in γ -aminobutyric acid. This is in agreement with the fact that glutamine may be considered as a storage compound for excessive amounts of ammonia which, in the case of normal plants, had been used up for

protein synthesis. The high content of γ -aminobutyric acid may have been due to the fact that it is not built into protein.

b) Experiments with spinach plants (July and August 1955). As in the experiment with cauliflower, three types of plants were used in this case, viz. those with severe symptoms of molybdenum deficiency, those with slight symptoms, and healthy plants. Analyses were carried out in samples harvested approximately 8 and 25 hours after addition of molybdate. The results accord with those of the previous experiment in respect of the response to molybdenum of the amino acids of the non-protein fraction. Glycine, however, which was formed in large amounts in molybdenum-deficient cauliflower plants upon addition of molybdate, was not found – or occurred in traces only – under similar conditions in spinach plants. In as little as 8 hours the addition of molybdate to the soil in which the Mo-deficient spinach plants were growing caused great increases (between 30 and 250 per cent) in the contents of 7 amino compounds. Hence no conclusions can be drawn as to the sequence of formation of these compounds. A second experiment with molybdenum-deficient spinach plants was therefore carried out in which the amino acids were assayed 2, 4, 6, and 24 hours after the addition of molybdate to deficient plants (Table IV).

It will be seen that 2 hours after molybdenum treatment the contents of free glutamic acid and glutamine showed the most pronounced rises. Serine, α -alanine, and aspartic acid had only slightly increased. Four hours after molybdenum application, the increase in content of free serine, α -alanine, aspartic acid, glutamic acid and particularly glutamine had continued. Twenty-four hours after molybdenum treatment glutamine had increased enormously. The amino acid aspartic acid now showed the highest rise, and in addition protein synthesis had also started. When a comparison is made between molybdenum-deficient plants 24 hours after treatment with molybdenum and those grown with adequate amounts of molybdenum during their entire development, it is seen that the protein content of the latter plants is considerably higher whereas their content of the free amino compounds, including glutamine, is much lower.

c) Experiment with tomato plants (August 1954). In this experiment a comparison was made between the synthesis of amino acids in molybdenum-deficient plants dressed with molybdate and

TABLE IV

Effect of the addition of molybdate to molybdenum-deficient spinach plants on the content of free amino acids in their leaves (mg amino acid per g dry matter) *										
Amino compound **	Plants with basic dressing only								Normal plants (Mo before sowing)	
	2 h after addition of		4 h after addition of		6 h after addition of		24 h after addition of		1 h	25 h
	H ₂ O	Mo	H ₂ O	Mo	H ₂ O	Mo	H ₂ O	Mo	(H ₂ O)	(H ₂ O)
Serine †	0.6	0.9	0.5	1.4	0.5	1.9	0.8	1.9	0.7	0.4
α-Alanine	0.5	0.7	0.7	1.4	0.9	1.7	0.9	2.2	0.5	0.4
Threonine ††	0.3	0.3	0.3	0.4	0.3	0.5	0.5	0.5	0.4	0.3
Valine	0.2	0.3	0.2	0.3	0.3	0.3	0.1	0.1	0.3	0.2
Leucines	0.3	0.2	0.3	0.4	0.3	0.5	0.2	0.8	0.2	0.1
Aspartic acid	1.5	1.7	1.4	3.2	1.7	3.4	1.1	6.4	2.7	2.2
Glutamic acid	4.7	5.7	5.4	7.0	5.6	7.0	4.8	8.0	5.8	3.4
γ-Aminobutyric acid	0.8	0.6	0.7	1.3	1.0	1.0	1.2	1.6	0.6	0.8
Glutamine	0.4	1.1	0.4	3.2	0.6	5.6	0.9	10.1	1.4	0.8
Soluble non-protein-N ++	11.4	9.3	8.9	10.6	9.2	10.4	8.3	12.9	8.8	7.5
Protein-N ++	27.7	28.0	26.7	26.1	26.6	26.3	28.0	30.0	43.0	41.8
NO ₃ -N++	28.8	30.7	37.4	31.9	36.1	31.0	30.6	23.9	2.8	2.9

* Averages of quadruplicate values.

** Proline was found in traces only; cystine + cysteine, methionine + methionine sulfoxide, tyrosine, arginine, lysine, and asparagine were absent; phenylalanine, tryptophane, and histidine were not determined.

† Serine was contaminated with a trace of glycine.

†† The spot of threonine on the chromatogram may have consisted partly of homoserine (cf Ref. 6²⁹).

++ mg per g of dry tissue.

that in nitrogen-deficient plants dressed with nitrate. For this purpose the plants were grown in a molybdenum-deficient soil, in one case in the absence of added molybdenum but supplied with nitrate and in the second in the presence of added molybdenum but without nitrate. Amino-acid analyses were carried out in samples harvested 2, 3, 6, and 8 days after the addition of molybdate to the molybdenum-deficient plants and of nitrate to the nitrogen-deficient plants. Although the first harvests were made relatively late, some interesting conclusions can be drawn from the amino-acid analyses (Table V). As with the cauliflower and spinach plants, asparagine was not found among the free amino compounds in tomato leaves. Glutamine showed the most striking rise two days after the application of molybdenum to molybdenum-deficient plants and of

TABLE V

Effect of the addition of molybdate to molybdenum-deficient and of nitrate to nitrogen-deficient tomato plants on the content of free amino acids in their leaves (mg amino acid per g dry matter). *										
Plants	Mo-deficient					Nitrogen-deficient				
Treatment.	No Mo added	5 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ added				No KNO_3 added	1.5 g KNO_3 added			
Days after treatment	1	2	3	6	8	1	2	3	6	8
<i>Amino compound **</i>										
Glycine	tr	tr	0.9	1.5	1.7	tr	tr	0.2	0.3	0.8
α -Alanine	0.4	2.8	1.0	1.1	1.2	0.1	0.5	0.6	0.9	1.3
Serine	tr	tr	0.7	0.4	0.6	tr	0.1	0.2	0.2	0.4
Threonine	tr	1.2	0.4	0.6	0.5	tr	tr	0.1	tr	0.4
Valine	tr	0.3	tr	0.3	0.2	tr	tr	tr	tr	0.2
Leucines	tr	0.5	tr	0.5	0.4	0.0	tr	tr	tr	0.2
Aspartic acid	0.7	1.4	0.9	0.9	0.5	0.2	0.5	0.7	0.6	0.5
Glutamic acid	1.3	3.2	1.9	0.8	0.6	0.2	0.2	0.6	0.5	0.5
Arginine	tr	2.2	tr	1.2	1.1	0.0	0.0	0.4	tr	0.0
γ -Aminobutyric acid	1.9	3.6	3.6	3.9	4.1	0.5	1.2	2.0	3.2	3.8
Glutamine	tr	6.6	1.9	3.6	3.3	tr	4.0	4.0	1.6	2.0

* Averages of quadruplicate values.

** Proline and asparagine were found in traces. Cystine + cysteine, tyrosine, methionine + methionine sulfoxide and lysine were absent. Phenylalanine, tryptophane, histidine, soluble non-protein-N, protein-N, and $\text{NO}_3\text{-N}$ were not estimated.

nitrate to nitrogen-deficient plants. In subsequent harvests its content decreased, apparently because it was used for protein synthesis. Glutamic and aspartic acids showed similar trends. γ -Aminobutyric acid, however, increased constantly, so that eight days after the addition of molybdate and nitrate this amino compound occurred in the highest amount. When a comparison is made between the molybdenum-deficient and the nitrogen-deficient plants it is seen that the latter were lower in free amino compounds than the former, and that the rise in content of these compounds was somewhat less upon the addition of nitrate than on the addition of molybdenum. A close resemblance may be observed, however, between the responses to molybdenum and nitrogen.

DISCUSSION

To study the mechanism of nitrogen fixation, experiments with labelled nitrogen have been carried out ^{1 19 30 31}. In these experi-

ments isolation and subsequent analysis for N^{15} of the nitrogenous compounds, involved in these reactions, are essential procedures. Isolation of adequate quantities of these compounds to enable testing for N^{15} is not an easy task, however.

The method described in the present paper enables the study of the mechanism of nitrogen fixation and of nitrate assimilation without isolation of the nitrogenous compounds. As a result of this the estimation of the formation of much smaller quantities of these compounds is possible than in the case of the isotopic technique.

The procedure depends on the addition of small amounts of molybdate to molybdenum-deficient leguminous plants. The latter are provided with normal or even more than normal numbers of nodules which fix the atmospheric nitrogen inadequately or not at all, however. The enzymes required for N_2 -fixation presumably are present in approximately normal amounts; their inactivity as far as nitrogen fixation is concerned apparently depends on the lack of molybdenum only. Addition of molybdate to the molybdenum-deficient plants restores the activity of the nitrogen-fixing enzymes within some hours (cf Table II). Estimation of free amino acids by chromatographic methods in the nodules, different periods of time after the addition of molybdenum, enables the study of the sequence of reactions in nitrogen fixation. Similarly the effect of added molybdenum on other metabolic processes may be studied.

In the present investigation amino-acid analyses have been carried out in two experiments with nodules from molybdenum-deficient lucerne plants and in one with clover nodules. These analyses showed that the number of amino acids occurring in the free state in these nodules is restricted. In molybdenum-deficient lucerne, glutamic acid was the only amino acid which occurred in the nodules in amounts higher than 1 mg per g of dry matter; in addition relatively large amounts of asparagine were found. Addition of molybdate brought about a rise in the contents of free glutamic acid, α -alanine, γ -aminobutyric acid, and asparagine. A number of other amino acids including glycine, serine, valine, the leucines, and aspartic acid were also slightly increased. In the second experiment with lucerne nodules the first analyses were carried out 5 hours after the addition of molybdate. During the first few hours glutamic-acid content showed the most pronounced rise. This may indicate that glutamic dehydrogenase is the most important enzyme in

synthesizing amino acids from fixed nitrogen in lucerne nodules, a conclusion which confirms the results of Allison and Burris¹ obtained with labelled N_2 in *Azotobacter vinelandii*. The fact that the contents of α -alanine, aspartic acid, and γ -aminobutyric acid had also increased slightly a few hours after addition of molybdate, indicates the existence either of a very active transaminase system or, in addition to glutamic dehydrogenase, of enzyme systems which catalyze the direct synthesis of α -alanine and aspartic acid. The increase of the asparagine content started relatively late but continued to very high values ten days after the addition of molybdate. This shows that asparagine must be considered as a secondary assimilation product. Definite proof of the existence in nodules of leguminous plants of more than one primary amino-acid-synthesizing system awaits the application of appropriate inhibitors for eliminating transaminase activity.

The experiments with molybdenum-deficient cauliflower, spinach, and tomato plants have shown that synthesis of amino acids from accumulated nitrate started within a few hours after the addition of molybdate to the soil in which the plants were growing. Consequently, no conclusions concerning the sequence of formation of amino acids can be drawn from the experiment with cauliflower plants in which the first analyses were carried out 17 hours after the addition of molybdate (Table III). Responses of molybdenum-deficient cauliflower leaves to added molybdate differed from those of nodules of molybdenum-deficient lucerne and clover plants. Although glutamic-acid content had increased considerably, the rise of glutamine was more striking. Apparently the amide was formed readily from glutamic acid. Other amino acids which had increased considerably were glycine, α -alanine, serine, and aspartic acid. The rise of glycine content to very high levels is very remarkable, since this amino acid was practically absent in normal cauliflower leaves.

The experiment with spinach leaves provided evidence that glutamic acid is the primary amino acid to be synthesized. This may be concluded from the fact that two hours after the addition of molybdate to Mo-deficient plants, glutamic acid and particularly glutamine had increased considerably, but aspartic acid and α -alanine had increased only slightly.

SUMMARY

A study was made of the sequence in which amino acids are formed during nitrogen fixation by root nodules of lucerne and clover plants, and during nitrate assimilation by cauliflower, spinach, and tomato plants. For this investigation, use was made of the fact that both nitrogen fixation and nitrate reduction require molybdenum as an essential element. In the absence of adequate amounts of molybdenum, leguminous plants are well supplied with nodules but the latter are unable to fix atmospheric nitrogen. As a result of this, the content of free amino acids in molybdenum-deficient nodules is very low. By adding small amounts of molybdate to Mo-deficient plants and estimating the amino acids in the nodules different periods of time after the application of molybdenum, information was gained concerning the synthesis of amino acids. The same principle was used for studying nitrate assimilation in cauliflower, spinach and tomato plants.

Some hours after the application of molybdate to the soil in which Mo-deficient lucerne plants were growing, the content of free glutamic acid showed a pronounced rise. The contents of α -alanine, aspartic acid, and γ -aminobutyric acid had increased slightly. The synthesis of asparagine, however, which is the most abundant amino compound in normal nodules, started more than 24 h after the application of molybdenum.

In the case of nitrate assimilation, response to added molybdenum was observed two hours after the addition of molybdate to the soil in which molybdenum-deficient spinach plants were growing. The contents of glutamic acid and glutamine showed the most pronounced increases; those of aspartic acid and α -alanine were only slightly increased. Asparagine was not found among the free amino compounds in leaves of cauliflower, spinach and tomato plants.

ACKNOWLEDGEMENT

The authors are much indebted to Dr. J. L. Harley, Department of Botany, University of Oxford, for reading and improving the text of this paper.

Received July 9, 1958

REFERENCES

- 1 Allison, R. M. and Burris, R. H., Kinetics of fixation of nitrogen by *Azotobacter vinelandii*. J. Biol. Chem. **224**, 351-364 (1957).
- 2 Alten, F., Wandrowsky, B., and Hille, E., Die Bestimmung des Nitratstickstoffs in Pflanzensubstanzen als Nitroxylenol. Bodenk. u. Pflanzenernähr. **1**, 340-348 (1936).
- 3 Anderson, A. J., Molybdenum deficiency on a South Australian ironstone soil. J. Australian Inst. Agr. Sci. **8**, 73-75 (1942).

- 4 Anderson, A. J., Thomas, M. P., and Oertel, A. C., Plant responses to molybdenum as a fertilizer. Council Sci. Ind. Research Commonwealth Australia Bull. **198** (1946).
- 5 Arnon, D. J., Ichioka, P. S., Wessel, G., Fujiwara, A., and Woolley, J. T., Molybdenum in relation to nitrogen metabolism. I. Assimilation of nitrate nitrogen by *Scenedesmus*. *Physiol. Plantarum* **8**, 538-551 (1955).
- 6 Bakhuis, J. A., Homoserine in bleeding sap of pea plants. *Nature (London)* **180**, 713 (1957).
- 7 Birch-Hirschfeld, L., Über den Einfluss von Molybdän und Bodenextraktstoffen auf die N-Bindung von *Azotobacter chroococcum*. *Arch. Mikrobiol.* **3**, 341-361 (1932).
- 8 Bortels, H., Molybdän als Katalysator bei der biologischen Stickstoffbindung. *Arch. Mikrobiol.* **1**, 333-342 (1930).
- 9 Burema, S. J. and Wieringa, K. T., Molybdenum as a growth factor of *Azotobacter chroococcum*. *Antonie van Leeuwenhoek* **8**, 123-133 (1942).
- 10 Burk, D. and Lineweaver, H., The influence of calcium and strontium upon the catalysis of nitrogen fixation by *Azotobacter*. *Arch. Mikrobiol.* **2**, 155-186 (1931).
- 11 Green, M. and Wilson, P. W., Hydrogenase and nitrogenase in *Azotobacter*. *J. Bacteriol.* **65**, 511-517 (1953).
- 12 Ichioka, P. S. and Arnon, D. J., Molybdenum in relation to nitrogen metabolism. II. Assimilation of ammonia and urea without molybdenum by *Scenedesmus*. *Physiol. Plantarum* **8**, 552-560 (1955).
- 13 Kluyver, A. J. and Reenen, W. J. van, Über *Azotobacter agilis* Beijerinck. *Arch. Mikrobiol.* **4**, 280-300 (1933).
- 14 Lee, S. B. and Wilson, P. W., Hydrogenase and nitrogen fixation by *Azotobacter*. *J. Biol. Chem.* **151**, 377-385 (1943).
- 15 Man, Th. J. de, De samenstelling van het graseiwit. *Landbouwk. Tijdschr.* **54**, 739-748 (1942).
- 16 Mulder, E. G., Importance of molybdenum in the nitrogen metabolism of microorganisms and higher plants. *Plant and Soil* **1**, 94-119 (1948).
- 17 Mulder, E. G., Molybdenum in relation to growth of higher plants and microorganisms. *Plant and Soil* **5**, 368-415 (1954).
- 18 Mulder, E. G. and Bakema, K., Effect of the nitrogen, phosphorus, potassium and magnesium nutrition of potato plants on the content of free amino-acids and on the amino-acid composition of the protein of the tubers. *Plant and Soil* **7**, 135-166 (1956).
- 19 Newton, J. W., Wilson, P. W., and Burris, R. H., Direct demonstration of ammonia as an intermediate in nitrogen fixation by *Azotobacter*. *J. Biol. Chem.* **204**, 445-451 (1953).
- 20 Nicholas, D. J. D., The function of trace metals in the nitrogen metabolism of plants. *Ann. Botany* **21**, 587-598 (1957).
- 21 Nicholas, D. J. D. and Nason, A., Molybdenum and nitrate reductase. II. Molybdenum as a constituent of nitrate reductase. *J. Biol. Chem.* **207**, 353-360 (1954).
- 22 Schröder, M., Die Assimilation des Luftstickstoffs durch einige Bakterien. *Zentrbl. Bakteriell. Parasitenk.* **85**, 177-212 (1931-1932).
- 23 Shug, A. L., Hamilton, P. B., and Wilson, P. W., Hydrogenase and nitrogen fixation. In: *Inorganic Nitrogen Metabolism*, p. 344-360; ed. by W. D. McElroy and Bentley Glass; Hopkins Press, Baltimore, U.S.A. 1956.
- 24 Shug, A. L., Wilson, P. W., Green, D. E., and Mahler, H. R., The role of molybdenum and flavin in hydrogenase. *J. Am. Chem. Soc.* **76**, 3355-3356 (1954).
- 25 Steinberg, R. A., Relation of accessory growth substance to heavy metals including molybdenum, in the nutrition of *Aspergillus niger*. *J. Agr. Research* **52**, 439-448 (1936).
- 26 Steinberg, R. A., Role of molybdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*. *J. Agr. Research* **55**, 891-902 (1937).

- 27 Thompson, J. F. and Steward, F. C., Investigations on nitrogen compounds and nitrogen metabolism in plants. II. Variables in two-directional paper chromatography of nitrogen compounds: A quantitative procedure. *Plant Physiol.* **26**, 421-440 (1951).
- 28 Thompson, J. F., Zacharius, R. M., and Steward, F. C., Investigations on nitrogen compounds and nitrogen metabolism in plants. I. The reaction of nitrogen compounds with ninhydrin on paper; A quantitative procedure. *Plant Physiol.* **26**, 375-397 (1951).
- 29 Wieringa, K. T. and Bakhuis, J. A., Chromatography as a means of selecting effective strains of Rhizobia. *Plant and Soil* **8**, 254-262 (1957).
- 30 Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., Isolation of the key intermediate in biological nitrogen fixation by *Clostridium*. *J. Biol. Chem.* **191**, 295-298 (1958).
- 31 Zelitch, I., Wilson, P. W., and Burris, R. H., The amino acid composition and distribution of N^{15} in soybean root nodules supplied N^{15} -enriched N_2 . *Plant Physiol.* **27**, 1-8 (1952).

THE EFFECT OF MOLYBDENUM AND NITROGEN DEFICIENCIES ON NITRATE REDUCTION IN PLANT TISSUES *

by E. G. MULDER, R. BOXMA **, and W. L. VAN VEEN

Laboratory of Microbiology, Agricultural University,
Wageningen, the Netherlands.

INTRODUCTION

The mechanism of nitrate reduction in fungi and higher plants has been cleared up by Nason and Evans^{3 8} and Nicholas and Nason¹¹. In experiments with purified cell-free extracts they found that the nitrate→nitrite reaction is catalyzed by an enzyme with either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as the prosthetic group and moreover molybdenum as a constituent. Reduced di- or triphosphopyridine nucleotide (DPNH or TPNH) are required as electron donor. The reduction sequence was established to be as follows:



Recent investigations by Nicholas and Scawin¹² and by Kinsky and MacElroy⁴ have shown that nitrate reductase requires inorganic phosphate for maximal activity. The latter authors furthermore found that their preparations of nitrate reductase isolated from *Neurospora* were able to reduce cytochrome c in the presence of TPNH.

In the reduction of nitrite to ammonia a number of enzymatic reactions are involved. Medina and Nicholas (see Nicholas⁹) have recently identified three metal-containing flavin enzymes in

* The major part of this work was carried out by the authors at the Institute for Soil Fertility, Groningen.

** Institute for Soil Fertility, Groningen.

cell-free extracts of *Neurospora*, viz. a nitrite reductase which reduces nitrite to hyponitrite, a hyponitrite reductase which reduces hyponitrite to hydroxylamine, and a hydroxylamine reductase which converts hydroxylamine to ammonia. There is an indication that the first two of these TPNH- or DPNH-requiring enzymes depend on the presence of copper or iron, and the third on manganese.

In the present study nitrate-reducing capacities of molybdenum-deficient cauliflower, spinach, and tomato plants were estimated at different periods of time after the addition of molybdate to the soil in which the plants were growing. In a number of experiments the molybdenum-deficient leaves were infiltrated with dilute solutions of sodium molybdate.

For comparison, the effect of the addition of nitrate or ammonium sulphate on the nitrate-reducing capacity of nitrogen-deficient cauliflower plants, well-supplied with molybdenum, was studied.

EXPERIMENTAL

Protein- and soluble non-protein-nitrogen were determined as described in a previous paper⁷.

Nitrate was estimated according to the xyleneol method¹.

Nitrate-reducing capacity of plant tissues was measured by using coarsely cut fragments of approximately 2 to 3 by 2 to 3 mm. One-gram portions were transferred to Thunberg tubes and provided with 5 ml 0.06 *M* phosphate buffer (pH 7.1), 1 ml 0.1 *M* malic acid neutralized with sodium hydroxide, 1 ml 0.1 *M* KNO₃ and 2 ml H₂O. After evacuation of the tubes air was slowly admitted to promote the uptake of the solution by the tissue fragments. Then the tubes were re-evacuated and incubated at 37°C for half an hour. The nitrite formed by the tissue fragments and released into the solution was determined according to the Griess-Romijn method¹³ with a mixture of α -naphthylamine and sulphanilic acid.

Enzyme activity was stopped by adding 1 ml of acetic acid and the solution was cleared with 3 ml of a saturated solution of (NH₄)₂SO₄. Blank values, obtained by adding 1 ml of acetic acid and 3 ml of saturated (NH₄)₂SO₄ to the complete mixture before evacuation, were subtracted.

To study the effect of molybdenum and nitrate supply to cauliflower, spinach, and tomato plants on nitrate-reducing capacity of the tissues, the plants were grown as described in a previous paper⁷ on molybdenum-deficient soil either in the absence of molybdenum with added nitrate, or in the presence of molybdenum without nitrate. The effect of molybdenum was studied by adding molybdate to the soil or by infiltrating it into the leaves of

molybdenum-deficient plants by vacuum infiltration. Similar treatments were used for nitrogen in the study of the effect of nitrate.

The infiltration of molybdate and nitrate solutions was performed by transferring leaves or leaf fragments to small beakers containing these solutions which were placed in an exsiccator. After evacuation, air was slowly admitted and as a result the pores of the leaf tissues were filled with liquid. The leaves were then exposed to an air stream to reduce their moisture content to the original level and incubated for different periods of time at room temperature in a moist atmosphere.

Malic-dehydrogenase activity in leaf tissue was estimated by transferring 0.5 g of tissue fragments to Thunberg tubes and adding 1 ml 0.1 *M* malic acid neutralized with sodium hydroxide, 0.5 mg DPN in 0.5 ml H_2O , 0.2 ml 0.2 *M* KCN, 0.5 ml 0.035% methylene blue, and 7.8 ml 0.06 *M* phosphate buffer (pH 7.0). The Thunberg tubes were evacuated before adding the methylene-blue solution, air was slowly admitted, methylene blue added, tubes again evacuated and incubated at 37°C. Time of reduction of methylene blue was taken as a measure of malic-dehydrogenase activity ⁵.

Catalase activity was estimated as follows. 0.2 to 0.5 g of ground plant material was transferred with 10 ml of 1/15 *M* phosphate buffer, pH 7.0, to a 1-l Erlenmeyer flask fitted with a small, calibrated separatory funnel containing a 6% solution of H_2O_2 in a similar phosphate buffer. The flask was further connected with a calibrated "Bunte" burette, filled with H_2O . Ten ml of H_2O_2 solution was added to the macerate while the flask was in connection with the atmosphere via the three-way stopcock of the burette.

Immediately after mixing the H_2O_2 and the plant extract, the stopcock was turned so that the amount of O_2 evolved by the catalase activity could be measured by the displacement of water from the burette. Care was taken that the readings were made at atmospheric pressure in the Erlenmeyer flask. During the test the flask was shaken constantly.

RESULTS

1. *Effect of molybdenum, added to molybdenum-deficient cauliflower, spinach, and tomato plants, on nitrate-reducing capacity of these plants*

a) Nitrate reduction in molybdenum-deficient plants upon addition of Na_2MoO_4 to the soil.

Cauliflower plants. Estimations of nitrate-reducing capacity were carried out in leaf fragments of molybdenum-deficient plants approximately 4½, 9¾, and 24¼ hours after application of 2.5 mg molybdate to 1 kg of the molybdenum-deficient soil in which the plants were growing. As will be seen from Table I, A, a twentyfold increase of nitrate-reducing capacity was found after 4½ hours. After an incubation

TABLE I

Effect of added molybdenum on nitrate-reducing capacity of molybdenum-deficient plants.*					
Treatment of plants	Plant tissue	NO ₃ -reducing capacity **	Treatment of plants	Plant tissue	NO ₃ -reducing capacity **
A			0 (continued)		
<i>Cauliflower</i>			0 Mo, 1 day Mo	Stem	4.0
0 Mo, control	Leaf	0.3		Seed leaf	2.3
	Stem	0.4	0 Mo, 3 days Mo	Leaf	3.4
0 Mo, 4½ h Mo	Leaf	6.3		Stem	5.2
	Stem	2.0		Seed leaf	3.0
0 Mo, 9¾ h Mo	Leaf	7.0	0 Mo, 6 days Mo	Leaf	0.9
	Stem	2.7		Stem	2.8
0 Mo, 24½ h Mo	Leaf	4.7		Seed leaf	1.1
	Stem	2.7	+ Mo, control	Leaf	0.5
+ Mo, control	Leaf	3.1		Stem	1.2
	Stem	2.0		Seed leaf	0.7
B			D		
<i>Cauliflower</i>			<i>Tomato</i>		
0 Mo, ½ h H ₂ O	Leaf	1.0	0 Mo, control	Leaf	0.2
0 Mo, 2 h Mo	Leaf	2.9		Stem	1.3
0 Mo, 2 h H ₂ O	Leaf	1.1	0 Mo, 1 day Mo	Leaf	2.1
0 Mo, 4¾ h Mo	Leaf	6.3		Stem	2.4
0 Mo, 4¾ h H ₂ O	Leaf	1.3	0 Mo, 2 days Mo	Leaf	1.2
				Stem	2.4
C			0 Mo, 5 days Mo	Leaf	1.2
<i>Spinach</i>				Stem	1.8
0 Mo, control	Leaf	0.0	0 Mo, 7 days Mo	Leaf	0.5
	Stem	0.5		Stem	2.4
	Seed leaf	0.5			
0 Mo, 1 day Mo	Leaf	3.7			

* Averages values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, in % of dry matter, respectively, of:

Cauliflower: **A**, 0 Mo control, leaf: 3.2, 1.7, 2.2; stem: 1.5, 1.1, 3.9; + Mo control, leaf: 4.4, 1.4, 1.1; stem: 1.5, 1.1, 3.3. **B**, 0 Mo, H₂O, leaf: 3.3, 1.8, 2.4.

Spinach: 0 Mo control, leaf: 2.8, 1.6, 3.1; stem: 1.9, 1.4, 3.4; seed leaf: 2.3, 1.1, 3.0; + Mo control, leaf: 4.5, 1.6, 0.7; stem: 2.3, 1.5, 2.7; seed leaf: 2.8, 1.4, 1.5; 3 and 6 days after treatment NO₃-N of the leaves had decreased to 2.7 and 1.8% respectively.

Tomato: 0 Mo control, leaf: 3.9, 0.9, 2.3; stem: 1.6, 0.6, 3.8; 2, 5 and 7 days after Mo treatment NO₃-N of the leaves had decreased to 1.9, 0.7, and 0.5% respectively.

** Averages of duplicate values expressed as µg NaNO₂ formed per ½h per mg protein.

time of 9¾ h some further increase of nitrate-reducing power had occurred. After 24 hours considerably lower values were found in leaf fragments, but not in stem tissue.

In a subsequent experiment with cauliflower plants nitrate-

reductase activity was measured approximately two hours after the addition of molybdate to the soil in which the molybdenum-deficient plants were growing. In this case nitrate-reducing capacity of leaf fragments had increased to a threefold level of the control (Table I, B). Plants of the same series tested five hours after the addition of molybdenum, showed a much higher activity. Apparently under the conditions of these experiments (greenhouse, autumn, approximately 20°C) nitrate-reducing capacity in molybdenum-deficient cauliflower leaves reached its full activity 4 to 5 hours after the addition of molybdate to the soil in which the plants were growing.

Spinach plants. Estimations of nitrate-reducing capacity in leaf and stem fragments were carried out 1, 3, and 6 days after the application of molybdate. High values were observed 1 day after molybdenum treatment (Table I, C). Six days after the addition of molybdenum, the nitrate-reducing capacity of molybdenum-deficient plants had decreased to a level slightly higher than that of plants supplied with adequate amounts of molybdenum from the beginning of their development.

The molybdenum-deficient spinach plants were extremely rich in nitrate nitrogen; the highest value found, viz. 3.45%, corresponds with a nitrate content of approximately 25% of the dry matter. Three days after the addition of molybdenum, the nitrate contents of the leaves and seed leaves had clearly decreased whereas the protein values had increased.

Tomato plants. Two experiments with molybdenum-deficient tomato plants have been carried out. In the first one it was shown that 4½ hours after the addition of molybdate to the soil, it had been taken up by the plants and had affected a pronounced rise in the nitrate-reducing capacity of the leaves.

In the second experiment, carried out in the second part of August 1954, nitrate-reductase activity was determined in leaves, stems and roots, 1, 2, 5, and 7 days after the addition of molybdenum. The highest value for leaf tissue was found 1 day after the application of molybdenum. After longer incubation times lower values were found (Table I, D). These results are in agreement with those of the preceding experiment with spinach plants. In stem tissue such variation did not occur.

b) Nitrate reduction in molybdenum-deficient plants upon infiltration with sodium molybdate.

Cauliflower plants. One half of molybdenum-deficient leaves was infiltrated with a solution containing 25 ppm of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, the other half with H_2O . It was shown that two hours after completion of the infiltration with molybdate, the nitrate reducing capacity had reached its maximal value (Table II, A).

Spinach plants. A comparison was made between nitrate-

TABLE II

Effect of infiltrated molybdate on nitrate reduction in Mo-deficient cauliflower, spinach, and tomato leaves.*					
Treatment of plants and infiltrated solution	Incubation time, h	Nitrate-reducing capacity **	Treatment of plants and infiltrated solution	Incubation time, h	Nitrate-reducing capacity **
A <i>Cauliflower</i>			B (Spinach continued)		
0 Mo, H_2O	2	0.4	+ Mo, H_2O	1	3.1
0 Mo, Na_2MoO_4	2	5.8	+ Mo, H_2O	4.25	4.1
0 Mo, Na_2MoO_4	4	4.2	+ Mo, Na_2MoO_4	1	3.3
			+ Mo, Na_2MoO_4	4.25	4.5
B <i>Spinach</i>			C <i>Tomato</i>		
0 Mo, no infiltration	0	0.6	0 Mo, H_2O	2	0.4
0 Mo, H_2O	1	2.2	0 Mo, Na_2MoO_4	2	2.4
0 Mo, H_2O	4.25	3.4	0 Mo, H_2O	4	0.4
0 Mo, Na_2MoO_4	1	4.3	0 Mo, Na_2MoO_4	4	4.9
0 Mo, Na_2MoO_4	4.25	8.4			
+ Mo, no infiltration	0	3.5			

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter: *Cauliflower*: 2.9, 1.4, 2.3. *Spinach*, 0 Mo: 3.2, 1.5, 2.3; + Mo: 4.3, 1.5, 0.7. *Tomato*: 3.3, 1.2, 2.2.

** Averages of duplicate values expressed as $\mu\text{g NaNO}_2$ formed per $\frac{1}{2}$ h per mg protein.

reducing capacities of molybdenum-deficient leaves infiltrated with a molybdate-containing solution (1 ml = 20 μg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) and H_2O , respectively. Estimations of nitrate-reducing capacities were made 1 and $4\frac{1}{4}$ h after infiltration. The results of this experiment recorded in Table II, B, demonstrate that 1 hour after completion of the infiltration with sodium molybdate, nitrate-reducing capacity has been restored only partly. At $4\frac{1}{4}$ h, a value was obtained

which was considerably higher than that of normal plants, even when the latter had been infiltrated with molybdate.

Infiltration with H_2O alone increased nitrate reduction of molybdenum-deficient plants considerably. In the case of normal plants this H_2O -effect was also observed, but to a smaller extent.

Tomato plants. Coarsely cut leaf fragments of molybdenum-deficient plants were infiltrated with H_2O or a solution containing 20 ppm of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. Two and four hours, respectively, after completion of the infiltration, the leaf fragments were transferred to Thunberg tubes and nitrate-reducing capacities estimated. Two hours after the infiltration with molybdate, the molybdenum-deficient leaf fragments had not yet obtained their maximal nitrate-reducing capacity (see Table II, C).

2. *Effect of the nitrogen supply to cauliflower, spinach, and tomato plants on nitrate-reducing capacity*

Since nitrate reductase presumably is an adaptive enzyme, it was suggested that plants inadequately supplied with nitrate nitrogen would possess a lower nitrate-reducing capacity than those well supplied with this nutrient. Experiments were therefore done with nitrogen-deficient plants which had been supplied, either by application to the soil, or by infiltration, with nitrate or an ammonium compound.

a) **Nitrate reduction in nitrogen-deficient plants upon addition of KNO_3 to the soil.** Experiments were carried out with spinach and tomato plants. These plants were grown in similar Neubauer jars as used in experiments with molybdenum, while the same molybdenum-deficient soil was employed. This soil was also dressed in the same way except that $2\frac{1}{2}$ mg Na_2MoO_4 was given per 1 kg of soil, and nitrate was omitted.

In the case of spinach, the nitrogen-deficient plants were characterized by a much reduced development as compared with plants normally supplied with nitrate; their leaf colour was, however, only of a slightly lighter green. 0.75 g KNO_3 was added per pot on 5 October 1954 and harvests were made after 1, 2, 3, and 6 days. In addition to nitrate-reducing capacity, estimations were made of protein-N, soluble non-protein-N and nitrate-N. The results of these experiments are recorded in Table III, A, those of a similar experiment with tomato plants in Table III, B. It will be seen that ad-

TABLE III

Effect of added KNO ₃ on nitrate-reducing capacity of nitrogen-deficient spinach and tomato plants.*					
Treatment of plants	Plant tissue	NO ₃ -reducing capacity **	Treatment of plants	Plant tissue	NO ₃ -reducing capacity **
A			B		
<i>Spinach</i>			<i>Tomato</i>		
0 N, control	Leaf	0.8	0 N, control	Leaf	0.0
	Stem	2.1		Stem	3.1
	Seed leaf	0.1		Stem	0.8
0 N, 2 days KNO ₃	Leaf	4.3	0 N, 1 day KNO ₃	Leaf	7.6
	Stem	3.7		Stem	0.4
	Seed leaf	2.5		Stem	4.1
0 N, 3 days KNO ₃	Leaf	3.3	0 N, 2 days KNO ₃	Leaf	1.9
	Stem	3.9		Stem	3.4
	Seed leaf	1.9		Leaf	1.1
0 N, 6 days KNO ₃	Leaf	2.1	0 N, 5 days KNO ₃	Stem	2.0
	Stem	2.8			
	Seed leaf	1.6			
+ N, control	Leaf	0.9	0 N, 7 days KNO ₃	Leaf	
	Stem	2.7		Stem	
	Seed leaf	0.5			

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter:

Spinach: 0 N control, leaf 3.7, 1.3, 0.1; stem 2.2, 1.7, 0.0; seed leaf 1.8, 0.9, 0.1; 2 and 6 days after KNO₃ treatment NO₃-N in the leaves increased to 0.6 and 0.7% respectively.

Tomato: 0 N control, leaf 1.9, 0.3, 0.0; stem 0.7, 0.5, 0.0; 2 and 7 days after KNO₃ treatment NO₃-N in the leaves had increased to 0.4 and 0.7% respectively.

** Averages of duplicate values expressed as $\mu\text{g NaNO}_2$ formed per $\frac{1}{2}$ h per mg of protein.

dition of nitrate to nitrogen-deficient plants brought about a pronounced rise of nitrate-reducing capacity. As with the molybdenum treatment of molybdenum-deficient plants this effect was most pronounced shortly after the application of the nitrate. After 6 or 7 days it was clearly reduced.

b) Effect of added nitrate and ammonium sulphate on nitrate-reducing capacity of nitrogen-deficient cauliflower leaves (28 October 1954). The nitrogen-deficient plants were considerably smaller than those dressed with nitrate, their leaf colour was of a slightly lighter green than normal, while the seed

leaves had died. Ten pots of this series were dressed with 5 ml of a solution containing 100 mg KNO_3 per ml. This solution was washed into the soil with 20 ml H_2O . A second series of ten pots was dressed with an equal amount of nitrogen in the form of $(\text{NH}_4)_2\text{SO}_4$, whereas a third series received H_2O only. At different periods of time after the addition of the nitrogenous compounds, plants were harvested and tested for nitrate-reducing capacity. The results of this experiment are recorded in Table IV. It will be seen that $4\frac{1}{2}$ h after the

TABLE IV

Effect of added KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ on nitrate-reducing capacity of nitrogen-deficient cauliflower leaves *					
Treatment of plants	Incubation time, hours	NO_3 -reducing capacity **	Treatment of plants	Incubation time, hours	NO_3 -reducing capacity **
H_2O , control	4.5	1.6	$(\text{NH}_4)_2\text{SO}_4$	23	2.7
KNO_3	4.5	4.3	H_2O , control	47	1.3
$(\text{NH}_4)_2\text{SO}_4$	4.5	3.2	KNO_3	47	6.4
H_2O , control	23	0.7	$(\text{NH}_4)_2\text{SO}_4$	47	3.4
KNO_3	23	5.1			

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter: 2.0, 0.7 and 0.0.

** Averages of duplicate values expressed as μg NaNO_2 formed per $\frac{1}{2}$ h per mg protein.

application of nitrate, the nitrate-reducing capacity of the leaves had considerably increased. After 1 and 2 days, respectively, this activity had still further increased. Plants dressed with ammonium sulphate also showed increased nitrate reduction though to a lesser extent than those treated with nitrate.

c) Infiltration of KNO_3 into nitrogen-deficient tomato leaves (1 November 1954). For this experiment plants with moderate symptoms of nitrogen deficiency were employed. Coarsely cut leaf fragments were infiltrated with a KNO_3 -containing solution (1 ml = 10 mg) and H_2O , respectively. $2\frac{2}{3}$ and 5 h after infiltration, nitrate-reducing capacities were determined. As may be seen from the results in Table V, A, nitrate-reducing power had considerably increased $2\frac{2}{3}$ h after infiltration with a nitrate-containing solution. After 5 h a further increase had taken place.

d) Effect of infiltrated KNO_3 , $(\text{NH}_4)_2\text{SO}_4$ and K_2SO_4 on nitrate-reducing capacity of nitrogen-deficient tomato plants. In order to investigate whether the nitrate-reducing

TABLE V

Effect of infiltrated KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ on nitrate-reduction in nitrogen-deficient tomato leaves.					
Treatment of plants and infiltrated solution	Incubation time, hours	Nitrate-reducing capacity **	Treatment of plants and infiltrated solution	Incubation time, hours	Nitrate-reducing capacity ***
A *			B		
0 N, H_2O	3	1.6	0 N, H_2O	3½	26
0 N, KNO_3	2½	2.9	0 N, KNO_3	3½	129
0 N, H_2O	5	1.0	0 N, $(\text{NH}_4)_2\text{SO}_4$	3½	16
0 N, KNO_3	5	4.1	0 N, K_2SO_4	3½	20

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter: 3.2, 0.8, 0.0; after infiltration $\text{NO}_3\text{-N}$ had increased to 0.6%.

** Averages of duplicate values expressed as $\mu\text{g NaNO}_2$ formed per ½ h per mg protein.

*** Averages of duplicate values expressed as $\mu\text{g NaNO}_2$ formed per ½ h per g fresh weight.

capacity could be restored by infiltration of the leaves with solutions of KNO_3 or $(\text{NH}_4)_2\text{SO}_4$, an experiment was carried out with N-deficient tomato leaves on 5 November 1954. The infiltrations were done with solutions of KNO_3 (1 ml = 10 mg), $(\text{NH}_4)_2\text{SO}_4$ (1 ml = 6.5 mg) and K_2SO_4 (1 ml = 8.6 mg). The temperature during the incubation was 22°C. Five hours after the completion of the infiltration, the leaves were cut into coarse fragments and nitrate-reducing capacity was estimated. As will be seen from the data of Table V, B, nitrate-reducing capacity was restored only if the leaves had been infiltrated with KNO_3 .

That the effect of infiltrated KNO_3 , in the preceding experiments, was due to the formation of an adaptive nitrate-reducing enzyme and not to an improved nitrate supply to an existing enzyme system, was concluded from an experiment using leaves of normal cauliflower plants grown in the presence of nitrate. These dark-green leaves contained no detectable nitrate and were assumed to have a normal enzyme content. Fragments of such leaves produced immediately - in an incubation period of half an hour - considerable quantities of nitrite when incubated in evacuated Thunberg systems in the presence of nitrate. Controls in the absence of added nitrate produced little nitrite and hence it must be concluded that this

substance originated by reduction of added nitrate. The absence of a lag phase in the development of reducing power suggests that nitrate reductase was initially present in these leaves grown on nitrate, whereas the delay in development of nitrate reduction in leaves grown in the absence of nitrate in previous experiments suggests adaptive enzyme formation.

e) Comparison of the effects of infiltrated KNO_3 on N-deficient and of infiltrated Na_2MoO_4 on molybdenum-deficient tomato plants. For this experiment tomato plants were grown in molybdenum-deficient soil a) with added molybdenum but without nitrate, b) with added KNO_3 but without molybdenum. The symptoms of nitrogen deficiency were less severe than those of molybdenum deficiency. The leaves of both series were harvested at 10 a.m. on 2 November 1954. The nitrogen-deficient samples were infiltrated with a solution of KNO_3 (1 ml = 10 mg); those from molybdenum-deficient plants were infiltrated with a solution containing 20 ppm $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. Control samples of both series were infiltrated with H_2O only. Nitrate-reducing capacities of coarsely cut leaf fragments were estimated by the usual procedure 2, 4, and 6 hours after infiltration (Table VI).

TABLE VI

Nitrate-reducing capacities of nitrogen-deficient tomato leaves * infiltrated with KNO_3 , and molybdenum-deficient tomato leaves * infiltrated with $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$					
Solutions infiltrated in N-deficient leaves	Incubation time, hours	Nitrate-reducing capacity **	Solutions infiltrated in Mo-deficient leaves	Incubation time, hours	Nitrate-reducing capacity **
H_2O	2	0.9	H_2O	2	0.1
KNO_3	2	2.6	Na_2MoO_4	2	1.0
H_2O	$4\frac{1}{2}$	1.8	H_2O	$4\frac{1}{2}$	0.1
KNO_3	$4\frac{1}{2}$	6.3	Na_2MoO_4	$4\frac{1}{2}$	2.8
H_2O	$6\frac{1}{2}$	1.1	H_2O	$6\frac{1}{2}$	0.3
KNO_3	$6\frac{1}{2}$	5.8	Na_2MoO_4	$6\frac{1}{2}$	3.4

* Average values for protein-N, soluble non-protein-N (except nitrate-N), and nitrate-N, respectively, in % of dry matter: 0 N: 3.1, 0.9, 0.0; after infiltration $\text{NO}_3\text{-N}$ had increased to 0.5%. 0 Mo: 3.1, 1.4, and 2.6%.

** Averages of duplicate values expressed as $\mu\text{g NaNO}_2$ formed per $\frac{1}{2}$ h per mg of protein.

From the results in Table VI it may be seen that two hours after completion of the infiltration the nitrate-reducing capacities of N-deficient plants had been restored to approximately 50 per cent and that of Mo-deficient plants to approximately 30 per cent.

3. Other factors affecting nitrate-reducing capacity in leaf fragments

a) Effect of cutting and grinding of leaves on nitrate reduction. To investigate whether the nitrite formation observed in leaf fragments in the preceding experiments must be attributed to intact or disrupted cells, experiments were carried out with leaf fragments of different sizes and with ground tissue. In the first experiment, spinach plants, grown in the presence of both nitrate and molybdate, were used. Leaves from these plants were cut into fragments of approximately 1 by 1, 2 by 2 and 5 by 5 mm, respectively, and tested for nitrate-reducing capacity as described above. The highest values were found in coarsely cut fragments, viz 52 μg NaNO_2 formed per 1 g fresh tissue per $\frac{1}{2}$ h at 37°C; fragments of 2 mm had formed 39 μg NaNO_2 under the same conditions and those of 1 mm only 15 μg . These results demonstrate that nitrate reduction in leaf fragments takes place mainly in undamaged cells. This was still more clearly shown when the leaf tissue was ground in a mortar. In the latter experiment a comparison was made between fragments cut to different sizes from normal cauliflower leaves and a macerate of the same amount of plant material. To investigate the possibility of nitrite binding or nitrite reduction, two samples of ground leaf tissue were provided with a certain amount of nitrite before evacuating the Thunberg tubes. The results of this experiment, recorded in Table VII, provide evidence that in addition to inactivation of the nitrate-reducing enzyme system, loss of nitrite occurs in leaf macerates. Nevertheless extracts of ground leaf tissue, infiltrated

TABLE VII

Effect of cutting and grinding of tomato leaves on nitrate-reducing capacity and nitrite removal.	
Treatment of leaf tissue	Nitrate-reducing capacity *
1. Whole leaves	51
2. Fragments of 5 by 5 mm	65
3. Fragments of 1-2 by 1-2 mm	22
4. Leaf macerate	8
5. Leaf macerate + 70 μg NaNO_2	51
6. Washed residue of macerate	10
7. Fragments of 5 by 5 mm, infiltrated with leachings of treatment 6	54

* Averages of duplicate values expressed as μg NaNO_2 formed per $\frac{1}{2}$ h per $\frac{1}{2}$ g fresh tissue.

into leaf fragments, hardly affected their nitrate-reducing power. Hence the depressing effect of grinding on nitrate reduction is not due to the presence of inhibitory substances.

The disappearance of nitrite in ground leaf tissue was shown more clearly in an experiment with spinach. Five grams of leaf macerate were provided with 300 μg NaNO_2 in addition to malic acid and phosphate buffer in the usual concentration. The mixture was incubated in an evacuated Thunberg tube for $1/4$ h at 37°C and subsequently analysed for nitrite. Only 122 μg of NaNO_2 were found which indicates a loss of approximately 60 per cent of the added nitrite.

The effect of ground leaf tissue on nitrite apparently is partly chemical and partly enzymatic. This was concluded from an experiment in which a comparison was made between fresh and boiled samples of a macerate of cauliflower leaves. 0.7 g of this macerate supplied with 250 μg of NaNO_2 under the usual conditions brought about a loss of 139 μg of NaNO_2 in $1/2$ h at 37°C when used in the fresh state and of 103 μg when it had been boiled. It is also possible that the effect of boiling is due to loss of nitrite-binding by the protein.

b) Effect of added yeast extract on nitrate reduction in leaf macerate. If it is assumed that nitrate reduction in leaf tissue is brought about by the activity of nitrate reductase, it can be suggested that either desorganization of the enzyme system or lack of substrate (DPNH or TPNH) were responsible for the inactivation of the nitrate-reducing capacity in disrupted cells. Therefore a few experiments with yeast extract (which is a source of FAD) have been carried out.

For the preparation of yeast extract, 25 g of dried "Engedura"-yeast were rubbed in a mortar, dissolved in 100 ml boiling water, and boiled for a few seconds. After cooling, the solution was centrifuged and the clear yellow centrifugate used for the enzyme tests.

In the first two experiments with macerates of normal spinach leaves a remarkable effect of yeast extract on restoring nitrate reduction was observed (see Table VIII).

In the case of leaf fragments, yeast extract had no effect on nitrate reduction.

In subsequent experiments with spinach and cauliflower leaves the effect of added yeast extract was considerably less pronounced

TABLE VIII

Effect of yeast extract on nitrate-reducing capacity of ground spinach leaves			
First experiment		Second experiment	
Treatment	NO ₃ - reducing capacity*	Treatment	NO ₃ - reducing capacity*
0.5 g leaf macerate	3	0.5 g coarsely cut leaf fragm.	61
0.5 g l.m. + 1 ml yeast extract, added before grinding	30	dito + 1 ml yeast extract	65
0.5 g l.m. + 1 ml yeast extract added 20 min after grinding	19	0.5 g leaf macerate	4
1 ml yeast extract	4	dito + $\frac{1}{4}$ ml yeast extract	10
0.5 g l.m. + 60 μ g NaNO ₂	29	dito + 1 ml yeast extract	33
		dito + 2 ml yeast extract	24
		dito + 51 μ g NaNO ₂	22
		1 ml yeast extract	3
		2 ml yeast extract	4

* Averages of duplicate values expressed as μ g NaNO₂ formed per $\frac{1}{2}$ hour $\frac{1}{2}$ g fresh tissue at 37°C.

than in the above-mentioned two tests. This was presumably due to the fact that yeast extract itself caused a loss of added nitrite (2 ml yeast extract added to the usual reaction mixture in evacuated Thunberg tubes at 37°C reduced added nitrite in $\frac{1}{2}$ h from 130 to 30 μ g. This was not an enzymatic effect since it was also exerted by yeast extract which had been boiled twice.

When a correction was made for the nitrite-decomposing effect of both leaf macerate and yeast extract, a small but consistent nitrate reduction was observed in the leaf-macerate-yeast-extract mixtures of the later experiments.

c) Effect of illumination on nitrate-reducing capacity. A comparison was made between cauliflower plants kept in the dark or illuminated, for 3 hours, in a thermostate of approximately 20°C. No difference in nitrate-reducing capacity of leaf fragments was observed.

In a subsequent experiment, cauliflower plants, kept for two days in the dark at 20°C, showed a somewhat reduced nitrate-reducing capacity as compared with that of plants kept under greenhouse conditions (27 and 32 μ g of NaNO₂ formed per 1 g of leaf fragments per $\frac{1}{2}$ h, respectively). When the plants were kept first in the dark for one day and then exposed to light in the greenhouse for one day an increased nitrite production was found, viz 45 μ g NaNO₂ per 1 g of leaf fragments.

4. *Effect of molybdenum and nitrogen supply of cauliflower, spinach, and tomato plants on malic-dehydrogenase activity*

If it is assumed that DPNH or TPNH is used as the substrate in nitrate reduction, then a dehydrogenase activity is required to keep these nucleotides in the reduced state. The importance of dehydrogenase activity for nitrate reduction in leaf fragments of tomato plants was demonstrated in an experiment in which malic acid was omitted from the usual reaction mixture. Thirty-three μg of NaNO_2 were formed per $\frac{1}{2}$ g of leaf fragments against 47 μg when 1 ml 0.1 *M* malic acid had been added and 43 μg in the presence of 1 ml 0.1 *M* succinic acid. The fact that both malic and succinic acid affected nitrite formation, indicates that both malic and succinic dehydrogenases were active.

Malic-dehydrogenase activity was estimated according to the above-mentioned method in a number of tissues which were also tested for nitrate-reducing capacity. Table IX gives the results of

TABLE IX

Effect of molybdenum supply to spinach and cauliflower plants on malic-dehydrogenase and nitrate-reductase activities					
Spinach leaves			Cauliflower leaves		
Treatment	Malic dehydrogenase *	Nitrate-reductase **	Treatment	Malic dehydrogenase *	Nitrate-reductase **
0 Mo	35 (57)	9	0 Mo	61 (81)	15
0 Mo, 5 h after addition of Na_2MoO_4			0 Mo, 2 h after addition of Na_2MoO_4		
	53 (75)	74		31 (>160)	42
+ Mo	37 (53)	60	0 Mo	86 (>200)	17
			0 Mo, 4 $\frac{1}{2}$ h Na_2MoO_4	53 (>140)	99
			0 Mo	70 (>120)	18

* Averages of duplicate values. Activity expressed as time (in minutes) required for complete reduction of added methylene blue by $\frac{1}{2}$ g leaf fragments. In parentheses, values obtained when KCN was omitted from the reaction mixture.

** Averages of duplicate values expressed as μg NaNO_2 formed per $\frac{1}{2}$ h per g fresh tissue.

two experiments in which the effect of molybdenum supply on malic-dehydrogenase activity was estimated. For comparison nitrate-reducing capacities are recorded. It will be seen that in the case of spinach leaves no difference in malic-dehydrogenase activity

was observed between molybdenum-deficient and normal leaf tissue, while added molybdate had a depressing effect on activity. In cauliflower leaves, however, added molybdenum clearly promoted malic-dehydrogenase activity.

In the case of nitrogen-deficient cauliflower, added nitrate which promoted nitrate-reducing capacity (cf Table IV) did not affect malic dehydrogenase. Addition of $(\text{NH}_4)_2\text{SO}_4$ which favoured nitrate reduction slightly, adversely affected malic dehydrogenase (Table X).

TABLE X

Effect of added KNO_3 and $(\text{NH}_4)_2\text{SO}_4$ on malic-dehydrogenase activity of cauliflower leaves.*			
Treatment	Malic dehydrogenase **	Treatment	Malic dehydrogenase **
0 N	14 (>90)	—	—
0 N, $4\frac{1}{2}$ h KNO_3	13 (>90)	0 N, $4\frac{1}{2}$ h $(\text{NH}_4)_2\text{SO}_4$	30 (>90)
0 N	12 (117)	—	—
0 N, 23 h KNO_3	12 (105)	0 N, 23 h $(\text{NH}_4)_2\text{SO}_4$	21 (138)
0 N	11 (>100)	—	—
0 N, 47 h KNO_3	14 (>100)	0 N, 47 h $(\text{NH}_4)_2\text{SO}_4$	29 (>100)

* Nitrate-reducing capacities in Table IV

** Time (in minutes) required for complete reduction of added methylene blue; in parentheses: values obtained without KCN. Averages of duplicate values.

5. *Effect of molybdenum supply on catalase activity of cauliflower, spinach, and tomato plants*

a) Comparison of molybdenum-deficient and normal cauliflower and spinach leaves. Readings were made after different periods of time (see Fig. 1). It will be seen that catalase activity of molybdenum-deficient plants was considerably less than that of normal plants. As a result of this effect, pronounced differences in behaviour of molybdenum-deficient and normal plants were sometimes obtained when these plants were placed in dilute solutions of H_2O_2 . The former lost their turgescence within a short time whereas normal plants were not affected. These results were not consistent, however; in a number of cases, molybdenum-deficient spinach and tomato plants were not affected by H_2O_2 .

b) Effect of added molybdate. In a few further experiments the effect of added molybdate on catalase activity of molybdenum-deficient spinach and tomato plants was investigated. Although normal leaf tissue had a considerably higher catalase activity than

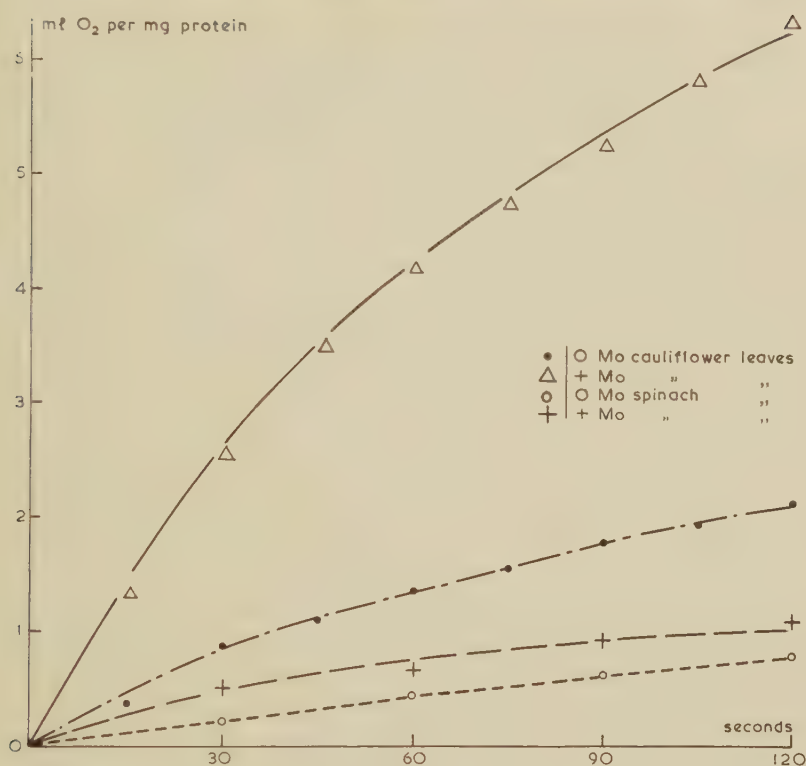


Fig. 1. The effect of molybdenum supply on the catalase activity (ml O₂ per mg protein) of cauliflower and spinach leaves. + Mo = 2.5 mg Na₂MoO₄·2H₂O per pot. Averages of quadruplicate values.

molybdenum-deficient, the effect of added molybdate, at least during the first days after addition, was slight. In contrast to nitrate-reducing capacity of Mo-deficient plants which was always restored within one day after molybdenum treatment, catalase activity six days after molybdenum treatment had not yet reached the value of normal plants.

DISCUSSION

The effect of molybdenum on nitrate reduction in cauliflower, spinach, and tomato leaves was studied by adding molybdate to Mo-deficient plants or by infiltrating leaves of the latter plants with molybdate-containing solutions. Nitrate-reducing capacities of the

tissues were determined under anaerobic conditions at 37°C * at different periods of time after molybdate had been provided; malate served as hydrogen donor. Under these conditions relatively large amounts of nitrite were formed and released into the solution in which the tissue fragments were suspended. Since omission of malic acid from the reaction solution reduced nitrite formation, while added DPNH had no promoting effect, it was assumed that electron donors (DPNH or TPNH) are available or are produced in the tissue fragments in adequate amounts for optimal nitrate reduction.

Under the conditions of the experiments, it took approximately 4 hours to restore nitrate-reducing capacity to molybdenum-deficient leaf tissue by the addition of sodium molybdate. It was remarkable that in most cases the nitrate-reducing capacities of Mo-deficient tissues provided with molybdate were higher than those of normal tissues well supplied with molybdenum from the beginning of their development. This indicates the accumulation of some stimulating compound in molybdenum-deficient plants.

The experiments with nitrogen-deficient cauliflower, spinach, and tomato plants provided evidence for the fact that nitrate reductase in these plants is an adaptive enzyme. The beneficial effect of ammonium sulphate on the nitrate reduction of nitrogen-deficient cauliflower plants, observed 4½ hours after its addition to the soil (Table IV) may have been due either to a very rapid nitrification of part of the added $(\text{NH}_4)_2\text{SO}_4$ or to the presence of some nitrate impurities in the ammonium-sulphate solution. That $(\text{NH}_4)_2\text{SO}_4$ itself has no effect on the nitrate-reducing capacity was concluded from the infiltration experiment recorded in Table V. These results are in agreement with those of Candela *et al.*² who found in cauliflower plants grown with ammonium sulphate as the nitrogen source, values for nitrate-reductase activity one-third and one-sixth of those observed in plants given nitrate or nitrite. Nicholas and Nason¹¹ obtained similar results with fungi.

The fact that a few hours after the addition of nitrate to the soil, the nitrate-reducing capacity of the leaves had increased to a large extent, makes it highly improbable that the restoration of the enzyme activity was due to the synthesis of entirely new protein from assimilated nitrate.

* At 20, 28 and 45°C lower values were obtained.

The experiments with leaf fragments of different sizes and with leaf macerates (Table VII) have provided evidence that the nitrate-reductase activity of the leaf fragments depends on the presence of undamaged cells. Grinding of the cells apparently inactivates the enzyme system; in addition it may disorganize the electron-donor system. Furthermore a loss of added nitrite was observed in leaf macerates. The fact that added yeast extract, which is a source of FAD, in some cases restored to a great extent the nitrate reduction of ground leaf tissue, but in other cases restored it only partly or not at all, may indicate that varying concentrations of reduced nucleotides were, at least partly, responsible for the irregular results of ground leaf tissues in the presence of yeast extract. That added FAD and TPNH are able to restore nitrate-reductase activity of ground leaf tissues may be concluded from the experiments of Evans and Nason³ and Candela *et al.*².

Illumination of cauliflower plants was found to have a depressing effect on nitrate-reducing capacity only if the plants were previously kept in the dark for a prolonged period of time. This is in agreement with the results of similar experiments of Candela *et al.*².

The effect of molybdenum on the nitrate-reducing capacity of leaf fragments as observed in the present investigation may have been due, in addition to its effect on nitrate reductase, to some extent to the influence on malic-dehydrogenase activity, *i.e.* on providing reduced nucleotides. This is concluded from the fact that malic-dehydrogenase activity in some experiments was clearly promoted by added molybdate. That the reduction of methylene blue, which was used as a measure for malic-dehydrogenase activity, was really brought about by the activity of this enzyme was concluded from the fact that omission of KCN from the reaction medium retarded methylene-blue decolourization to a large extent.

The effect of molybdenum on malic dehydrogenase was less consistent and far less pronounced than that on nitrate-reducing capacity, however.

A further effect of molybdenum, which had been noticed earlier^{6 10} is its influence on catalase activity. This is an indirect effect, however, and mainly depends, it is presumed, on newly synthesized protein. This is concluded from the slow rise in catalase activity upon addition of molybdate to molybdenum-deficient plants.

SUMMARY

The effect of molybdenum on the nitrate-reducing capacity of plant tissue was studied by using coarsely cut tissue fragments from plants grown in Mo-deficient soil and dressed or infiltrated with a molybdate-containing solution shortly before testing. The tissue fragments were suspended under anaerobic conditions in a nitrate-containing buffer solution with malate serving as a hydrogen donor. Formation of nitrite was used as a measure for the nitrate-reducing power. Very low values for nitrate-reducing capacity were found in molybdenum-deficient tissues. As little as four hours after the application of molybdenum, however, enzyme activity had reached its maximal value.

Cauliflower, spinach, and tomato plants, grown with inadequate amounts of nitrogen, showed also very low nitrate-reducing activities. Addition of nitrate to these plants, either by application to the soil or by infiltrating it into the leaves, resulted in a rapid rise in nitrate-reducing capacity. Ammonium sulphate, added to the soil, had some beneficial effect on nitrate reduction in the leaves of cauliflower plants but in the case of infiltration no effect was observed.

Evidence was obtained that the nitrate-reducing activity of leaf fragments depends on the presence of undamaged cells. Grinding of the cells inactivates the enzyme system; in addition it may desorganize the electron-donor system. Furthermore a loss of nitrite may occur in leaf macerates. Addition of yeast extract, which is a source of flavin adenine dinucleotide, a co-enzyme of nitrate reductase, restored in some cases the nitrate-reductase activities of leaf macerates to a large extent. In other cases its effect was slight or negligible.

Plants kept in the dark for a prolonged period of time showed lower nitrate-reducing capacities than those kept under normal light conditions.

In some experiments the malic-dehydrogenase activity of molybdenum-deficient plant tissues was found to be promoted by the addition of molybdate. This effect was less consistent and far less pronounced than that on nitrate-reducing capacity.

Molybdenum-deficient plant tissues usually have a lower catalase activity than plants supplied adequately with molybdenum.

Received July 9, 1958

ACKNOWLEDGEMENT

The authors are much indebted to Dr. J. L. Harley, Department of Botany, University of Oxford, for reading and improving the text of this paper.

REFERENCES

- 1 Alten, F., Wandrowsky, B., and Hille, E., Die Bestimmung des Nitratstickstoffes in Pflanzensubstanzen als Nitroxylol. *Bodenk. u. Pflanzenernähr.* **1**, 340-348 (1936).

- 2 Candela, M. I., Ficher, E. G., and Hewitt, E. J., Molybdenum as a plant nutrient. X. Some factors affecting the activity of nitrate reductase in cauliflower plants grown in different nitrogen sources and molybdenum levels in sand culture. *Plant Physiol.* **32**, 280-288 (1957).
- 3 Evans, H. J. and Nason, A., Pyridine nucleotide-nitrate reductase from extracts of higher plants. *Plant Physiol.* **28**, 233-254 (1953).
- 4 Kinsky, S. C. and MacElroy, W. D., *Neurospora* nitrate reductase: The role of phosphate, flavin and cytochrome c reductase. *Arch. Biochem. Biophys.* **73**, 466-483 (1958).
- 5 McShan, W. H., Dehydrogenase. Chapter in "Respiratory Enzymes"; ed. by H. A. Lardy, Burgess Publ. Comp., Minneapolis 2nd pr. 1950, p. 101-149.
- 6 Mulder, E. G., Molybdenum in relation to growth of higher plants and micro-organisms. *Plant and Soil* **5**, 368-415 (1954).
- 7 Mulder, E. G., Bakema, K., and Veen, W. L. van, Molybdenum in symbiotic nitrogen fixation and in nitrate assimilation. *Plant and Soil* **10**, 319-334 (1959).
- 8 Nason, A. and Evans, H. J., Triphosphopyridine nucleotide-nitrate reductase in *Neurospora*. *J. Biol. Chem.* **202**, 655-673 (1953).
- 9 Nicholas, D. J. D., The function of trace metals in the nitrogen metabolism of plants. *Ann. Botany* **21**, 587-598 (1957).
- 10 Nicholas, D. J. D., The effect of molybdenum deficiency on the catalase and peroxidase content of *Neurospora crassa*. *J. Gen. Microbiol.* **17**, 689-698 (1957).
- 11 Nicholas, D. J. D. and Nason, A., Molybdenum and nitrate reductase. II. Molybdenum as a constituent of nitrate reductase. *J. Biol. Chem.* **207**, 353-360 (1954).
- 12 Nicholas, D. J. D. and Scawin, J. H., A phosphate requirement for nitrate reductase from *Neurospora crassa*. *Nature (London)* **178**, 1474-1475 (1956).
- 13 Romijn, G., De colorimetrische bepaling van het salpeterigzuur. *Chem. Weekblad* **11**, 1115-1116 (1914).

EVALUATION OF LABORATORY INDEXES OF ABSORPTION OF SOIL PHOSPHORUS BY PLANTS: I *

by U. S. MOSER, W. H. SUTHERLAND, and C. A. BLACK **

The usual laboratory indexes of absorption of soil phosphorus by plants are subject to two important sources of bias: (1) the relative proportions of the several forms of soil phosphorus measured by the methods may differ from the relative proportions absorbed from these forms by plants; and (2) the intensity of action of the reagents on the phosphorus sources may not be independent of the nature of the soil because of reaction of the reagents with soil components other than the phosphorus sources.

Amer *et al.*¹ developed a method involving the use of an anion-exchange resin in an attempt to minimize the biases described above. The anion-exchange resin acts by removing the phosphorus from solution as the phosphorus is released from the solid phase. To this extent the mechanism is similar to that by which plants are thought to remove phosphorus from soils; this mechanism is fundamentally different from the means by which the usual acids, bases, complexing agents, or precipitating reagents cause the release of soil phosphorus. The resin employed is a strong-base type in chloride form that is only slightly buffered over the pH range of soils. In consequence the pH value of the soil is but little affected by addition of the resin.

The principal objective of the experimental work described in this paper is to compare the performance of the exchange-resin method of Amer *et al.*¹ with that of other methods in current use

* Journal Paper No. J-3452 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1183. Contribution from the Department of Agronomy.

** Graduate Students and Professor of Soils, respectively.

to see whether the supposed advantages of the resin method are sustained by actual trial. Since experience has shown that most methods will provide a good index of the absorption by plants of the phosphorus in similar soils, an attempt was made in the present investigation to give the various methods a rigorous test by applying them to dissimilar soils.

MATERIALS AND METHODS

Samples of 22 soils were collected from the United States through the cooperation of research workers in the respective states. The selection of sites was made on the basis of results of a series of phosphorus-fertilization experiments of uniform design that had been conducted in 1951 and 1952 *. Measurements of a labile fraction of inorganic phosphorus had been obtained for the original group of samples by the 0.025N HCl, 0.03N NH_4F extraction method of Bray and Kurtz ⁴. The samples were selected to give a low correlation between quantities of inorganic phosphorus extracted by this method and the quantities found by the *A*-value method of Fried and Dean ⁶. The samples used in the present investigation were collected from sites adjacent to the original experiments. Hence the samples are similar to, but not identical with, those of the original investigation. Table I lists the U.S. Department of Agriculture numbers of the original samples along with the Iowa numbers of the present samples, the soil type, location, and pH value (measured in a 1 to 2.5 soil to water suspension) and the individuals responsible for sending the samples.

The soil samples were taken from the plowed layer and were air dried before use. To measure the uptake of phosphorus from the soils by plants, Redbine-60 sorghum was grown on cultures of the various soils in the greenhouse. The cultures consisted of 400 g of soil mixed with 3600 g of quartz sand and supplied with ample quantities of nutrients other than phosphorus. Ten replicates were employed in a randomized-block design. The above-ground plant material was dried, weighed, and ground, and samples were analyzed for phosphorus by ashing with magnesium nitrate, dissolving the phosphorus in the residue with dilute hydrochloric acid, and measuring the phosphorus in an aliquot portion of the solution by the colorimetric method of Dickman and Bray ⁵.

Measurements of labile inorganic phosphorus were obtained in the laboratory by employing several different methods with samples of soil that were in the air-dry condition and with corresponding samples that had been

* Dean, L. A., Uniform phosphorus experiments: cooperative studies on the fertility status of soils. Unpublished multilithed manuscript. Bureau of Plant Industry, Soils and Agricultural Engineering, U.S. Dept. Agr., Beltsville, Maryland (1952).

Dean, L. A., Uniform phosphorus experiments in 1952. Unpublished multilithed manuscript. Bureau of Plant Industry, Soils and Agricultural Engineering, U.S. Dept. Agr., Beltsville, Maryland (1953).

incubated at room temperature for 7 days after the water content had been adjusted to the moisture equivalent by the method of Bouyoucos³. The laboratory methods employed were the "phosphate potential" method of Schofield⁸, as described by Aslyng², the 0.1N HCl, 0.03N NH₄F extraction method of Bray and Kurtz⁴, the 0.5M NaHCO₃ extraction method of Olsen *et al.*⁷, and the anion-exchange-resin extraction method of Amer *et al.*¹.

RESULTS AND DISCUSSION

The yields of phosphorus obtained in the sorghum plants grown in the greenhouse are shown in Table I. These figures were obtained by multiplying the yield of dry matter by the phosphorus percentage in the dry matter. The yields of dry matter and phosphorus percentages are given by Moser.* The results obtained from the laboratory measurements of soil phosphorus are shown in Tables II to VI.

The evaluation of the different laboratory methods is made on the basis of an analysis of variance of the regression of the yields of phosphorus in the plants on the measurements of soil phosphorus. The suitability of the various methods is assumed to increase with decreasing probability of obtaining an *F* ratio for the regression as large as or larger than that obtained experimentally if the true regression is zero in the population from which the sample is drawn. The probabilities are listed in Tables VIII and IX.

Comparing first the two types of samples employed, it is seen from Tables VIII and IX that the lower probability was obtained from the premoistened samples in 9 instances and from the initially dry samples in 20 instances. Hence there is no reason to conclude from the present work that premoistening is advantageous; in fact there may be some advantage from the use of dry samples. It must be noted, however, that in this investigation the entire sample of each soil was brought to the air-dry condition originally and a portion was incubated in a moist condition before analysis. A different type of results might have been obtained if the portion of the sample on which the plants were grown had been maintained in a moist condition.

In the Schofield-Aslyng method for assessing the phosphorus

* Moser, Ursula Siegrist, Correlation of soil and plant measurements of phosphorus availability. Ph. D. Thesis, Iowa State College, Ames, Iowa (1956).

TABLE I

Yield of phosphorus in sorghum plants grown in cultures of different soils

U.S.D.A. No.	Iowa No.	Source of soil sample		Soil type	Soil pH	Yield of phosphorus per culture, mg ***
		State	Contributor			
51526	F2814	S. Dak.	L. O. Fine	Barnes loam	6.75	18.1
51562	F2815	Wash.	W. P. Mortensen, R. L. Hausenbuiller	Cinebar silt loam	5.69	5.5
51563	F2816	Wash.	W. P. Mortensen, R. L. Hausenbuiller	Puyallup silt loam	5.30	17.8
51567	F2817	Utah	H. B. Peterson	Nibley clay loam	8.10	6.2
51569	F2818	Utah	H. B. Peterson	Draper loam	8.20	9.4
51572	F2819	Ala.	L. E. Ensminger	Lloyd clay loam	6.10	5.6
51574	F2820	Mont.	J. C. Hide, T. S. Aasheim, P. Pederson	Nunns silty clay	8.20	5.0
51576	F2821	N.Y.	D. J. Lathwell	Mardin silt loam	5.65	10.5
51582	F2822	Va.	C. I. Rich	Davidson clay loam	6.40	4.4
51583	F2823	Va.	C. I. Rich	Applying fine sandy l.	6.10	2.3
51585	F2824	Kans.	P. L. Brown	Yocemento silt loam *	6.40	17.2
51586	F2825	Mont.	J. C. Hide, T. S. Aasheim, P. Pederson	Huffine silt loam	7.50	18.4
51588	F2826	Nebr.	R. A. Olson	Carrington clay loam	6.45	2.5
51589	F2827	Nebr.	R. A. Olson	Moody silty clay	6.50	7.0
52592	F2828	Idaho	L. I. Painter, J. V. Jordan	Declo loam **	8.35	8.0
51615	F2829	Idaho	J. V. Jordan	Palouse silty clay l.	6.00	44.7
51616	F2830	Minn.	A. C. Caldwell	Waukegan silt loam	5.76	31.1
51627	F2831	Mich.	Kirk Lawton	Fox sandy loam	6.25	12.7
51633	F2832	Ga.	H. D. Morris	Lloyd clay loam	5.90	8.2
51634	F2833	Ga.	H. D. Morris	Orangeburg sandy l.	5.50	9.7
51652	F2834	N. Car.	A. Mehlich	Norfolk loamy sand	5.70	14.9
51745	F2835	Wash.	H. M. Reisenauer, R. L. Hausenbuiller	Walla Walla silt l.	6.21	33.2

* Formerly called Munjor silt loam

** From the 1952 experiments; all others from the 1951 experiments

*** The yield of phosphorus obtained in cultures without soil averaged 1.8 mg

status of soils, 20- and 40-g quantities of soil are shaken for 30 minutes with 50 ml of 0.01M CaCl_2 solution. The pH of the suspension and the inorganic phosphorus content of the clear extracts then are determined. The concentration of phosphorus in the extracts is plotted against the reciprocal of the quantity of soil employed. The straight line passing through the two experimental points is extrapolated to zero dilution. From the pH value, the calcium ion activity in the 0.01M CaCl_2 solution, and the phosphorus concentration at zero dilution, the "phosphate potential" is calculated.

The phosphate potential is equal to ($\frac{1}{2}\text{pCa} + \text{pH}_2\text{PO}_4$). Details of the methods of calculation are given by Aslyng ².

The data obtained in connection with the Schofield-Aslyng method are given in Tables II and III. Table VIII shows that the probabilities expressing the significance level of the regression of the yield of phosphorus in the plants on the phosphorus concentration in the 0.01M CaCl₂ extracts are of the order of 10⁻² to 10⁻³ and that the probabilities associated with the regression of yield of phosphorus on the phosphate potential are of the order of 10⁻³ to 10⁻⁴. Hence some improvement in prediction is associated with use of the phosphate potential in place of the concentrations in the 0.01M CaCl₂ extracts.

TABLE II

Phosphorus extracted from samples of different soils by 0.01M CaCl ₂ solution according to the Schofield-Aslyng method						
Soil No.	Initially dry samples			Premoistened samples		
	P concentration in 50 ml of 0.01M CaCl ₂ solution, ppm		Estimated concentration of P in soln. at zero dilution, micromols per liter	P concentration in 50 ml of 0.01M CaCl ₂ solution, ppm		Estimated concentration of P in soln. at zero dilution, micromols per liter
	20-g soil sample	40-g soil sample		20-g soil sample	40-g soil sample	
F2814	0.043	0.118	2.42	0.286	0.640	11.43
F2815	0.068	0.280	6.84	0.003	0.013	0.32
F2816	0.230	0.598	11.88	0.035	0.070	1.13
F2817	0.127	0.310	5.91	0.095	0.251	5.04
F2818	0.145	0.464	10.30	0.456	1.129	21.73
F2819	0.039	0.086	1.52	0.005	0.006	0.03
F2820	0.033	0.086	1.71	0.026	0.093	2.16
F2821	0.034	0.072	1.23	0.003	0.019	0.52
F2822	0.046	0.125	2.55	0.035	0.093	1.87
F2823	0.027	0.072	1.45	0.040	0.093	1.71
F2824	0.218	0.523	9.85	0.118	0.275	5.07
F2825	0.224	0.528	9.82	0.243	0.585	11.05
F2826	0.017	0.051	1.10	0.034	0.093	1.91
F2827	0.030	0.094	2.07	0.003	0.050	1.52
F2828	0.049	0.179	4.20	0.077	0.219	4.58
F2829	0.352	0.777	13.73	0.418	0.895	15.40
F2830	1.096	2.358	40.75	0.912	2.624	55.25
F2831	0.034	0.104	2.26	0.026	0.077	1.65
F2832	0.039	0.160	3.90	0.041	0.154	3.65
F2833	0.075	0.163	2.84	0.024	0.053	0.94
F2834	0.379	0.868	15.80	0.428	1.040	19.76
F2835	0.178	0.426	8.01	0.171	0.397	7.30

TABLE III

Soil pH, lime potential ($\text{pH} - \frac{1}{2} \text{pCa}$), and phosphate potential ($\frac{1}{2} \text{pCa} + \text{pH}_2\text{PO}_4$) according to the Schofield-Aslyng method						
Soil No.	Initially dry samples			Premoistened samples		
	pH	$\text{pH} - \frac{1}{2} \text{pCa}$	$\frac{1}{2} \text{pCa} + \text{pH}_2\text{PO}_4$	pH	$\text{pH} - \frac{1}{2} \text{pCa}$	$\frac{1}{2} \text{pCa} + \text{pH}_2\text{PO}_4$
F2814	6.15	4.98	6.94	6.50	5.33	6.33
F2815	5.08	3.91	6.43	5.35	4.18	7.77
F2816	4.70	3.53	6.19	5.00	3.83	7.21
F2817	7.50	6.33	7.13	7.50	6.33	7.20
F2818	7.60	6.43	6.97	7.60	6.43	6.64
F2819	5.50	4.33	7.09	5.60	4.43	8.80
F2820	7.60	6.43	7.75	7.60	6.43	7.64
F2821	5.05	3.88	7.18	5.75	4.58	7.57
F2822	5.80	4.63	6.88	5.75	4.58	7.02
F2823	5.50	4.33	7.11	5.20	4.03	7.04
F2824	5.80	4.63	6.30	5.85	4.68	6.59
F2825	6.90	5.73	6.53	6.85	5.68	6.46
F2826	5.85	4.68	7.25	5.90	4.73	7.02
F2827	5.90	4.73	6.98	6.10	4.93	7.13
F2828	7.75	6.58	7.48	7.50	6.33	7.24
F2829	5.40	4.23	6.13	5.50	4.33	6.09
F2830	5.15	3.98	5.66	5.45	4.28	5.53
F2831	5.65	4.48	6.93	6.15	4.98	7.10
F2832	5.30	4.13	6.68	5.80	4.63	6.73
F2833	4.90	3.73	6.81	5.15	3.98	7.30
F2834	5.10	3.93	6.07	5.45	4.28	5.98
F2835	5.61	4.44	6.38	5.50	4.33	6.41

The quantities of phosphorus extracted from the samples of the different soils by the 0.01N HCl, 0.03N NH_4F method of Bray and Kurtz and the 0.5M NaHCO_3 method of Olsen *et al.* are given in Table IV. As shown in Table VIII, the probabilities associated with the regression of the yield of phosphorus in the sorghum plants on the phosphorus extracted by the former method are in the range of 10^{-1} . These are the highest probabilities obtained, which means that the 0.1N HCl, 0.03N NH_4F extractant gave the poorest prediction of the yield of phosphorus. The probabilities obtained with the 0.5M NaHCO_3 extractant were in the range of 10^{-4} and 10^{-5} . Predictions of the yield of phosphorus from measurements by this method thus are more precise than those by the 0.01M CaCl_2 and 0.1N HCl, 0.03N NH_4F methods.

The quantities of phosphorus extracted from the different soils during different time intervals by the anion-exchange-resin method of Amer *et al.* are shown in Tables V and VI. The probabilities

TABLE IV

Phosphorus extracted from samples of different soils by the 0.5M NaHCO ₃ method of Olsen <i>et al.</i> and the 0.1N HCl-0.03N NH ₄ F method of Bray and Kurtz				
Soil No.	Phosphorus extracted, ppm of air-dry soil			
	Method of Olsen <i>et al.</i>		Method of Bray and Kurtz	
	Initially dry samples	Premoistened samples	Initially dry samples	Premoistened samples
F2814	39.2	35.6	67.9	64.2
F2815	18.9	16.5	8.6	12.6
F2816	58.6	56.0	478.5	187.9
F2817	16.5	17.2	577.3	572.4
F2818	31.7	28.0	356.9	208.6
F2819	9.5	10.4	10.3	12.9
F2820	7.5	6.0	243.6	137.3
F2821	20.0	15.8	31.2	32.6
F2822	5.0	6.9	8.3	9.3
F2823	3.9	1.7	8.7	5.5
F2824	30.4	28.5	175.5	92.8
F2825	32.0	32.2	212.2	114.6
F2826	4.6	3.7	5.4	8.2
F2827	12.5	11.3	74.5	72.9
F2828	18.1	16.3	45.1	36.6
F2829	45.8	45.6	266.1	144.5
F2830	71.5	75.1	340.8	187.9
F2831	27.0	22.0	74.5	55.6
F2832	11.9	14.4	42.9	29.7
F2833	20.6	20.5	62.0	50.6
F2834	45.8	41.0	403.9	147.1
F2835	25.5	27.4	186.6	105.0

(Table VIII) associated with the regressions of yield of phosphorus on the quantities of phosphorus extracted are in the range of 10^{-4} to 10^{-6} , with most of the values at a lower level than those obtained by the methods discussed above. Hence most of the predictions of yield of phosphorus obtained by the resin method are more precise than those obtained by the other methods.

The foregoing simple linear regressions provide a measure of conformity to the relationship, $y = a + bx$, where y is the yield of phosphorus in the plants grown in the greenhouse, x is the numerical value of the phosphorus measurement made on the soils in the laboratory, and a and b are constants. The simple linear model may be expanded to a more general case in which there are n independent variables. This model may be represented by the equation, $y = a + b_1x_1 + b_2x_2 + \dots + b_nx_n$, where y is the yield of phosphorus in the plants as before, $x_1, x_2, \dots, x_n$ are different

TABLE V

Soil No.	Phosphorus extracted from initially dry samples of different soils by the anion-exchange-resin method of Amer <i>et al.</i>											
	P extracted in ppm of air-dry soil during indicated length of time in hours											
	0.084	0.17	0.25	0.50	1	2	8	12	24	36	48	72
F2814	11.0	14.0	19.0	23.2	26.1	28.7	36.0	40.0	48.1	54.0	57.8	61.7
F2815	0.0	0.0	1.3	1.9	2.6	3.8	8.3	11.1	15.3	17.0	17.9	19.0
F2816	12.3	13.9	15.6	16.4	17.5	22.8	37.5	43.0	52.7	57.6	62.2	68.1
F2817	2.7	4.5	8.8	12.3	15.3	18.2	26.1	29.6	35.2	28.7	40.9	43.4
F2818	12.0	16.3	19.7	22.3	27.9	34.0	40.0	41.7	45.1	46.8	47.7	48.6
F2819	0.0	0.0	0.0	0.6	1.2	1.8	5.4	7.2	9.8	10.6	11.0	11.2
F2820	1.0	3.1	3.6	4.8	8.6	10.7	14.3	15.0	16.4	17.2	17.9	17.9
F2821	1.2	1.5	1.8	2.1	3.0	5.1	12.3	16.0	21.8	25.3	27.6	29.1
F2822	0.0	0.0	0.0	0.9	1.5	2.0	5.1	7.2	10.1	11.7	12.5	13.3
F2823	0.0	0.0	0.0	0.0	0.0	0.0	0.7	1.0	1.9	2.4	2.6	2.6
F2824	10.4	15.0	15.3	20.7	24.6	30.2	34.4	36.0	39.1	40.5	40.9	41.3
F2825	16.1	20.9	23.2	27.0	33.1	41.7	51.3	53.7	57.6	59.1	59.6	60.2
F2826	0.0	0.0	0.0	0.0	0.6	1.5	3.4	3.9	4.0	4.3	4.5	4.5
F2827	1.2	2.4	3.6	5.4	9.4	10.3	12.8	13.6	14.6	15.3	15.6	16.3
F2828	3.9	4.8	9.8	11.7	14.3	16.3	19.4	20.1	21.5	22.3	22.9	23.2
F2829	13.3	17.6	21.8	24.6	31.3	44.7	52.2	55.6	62.7	68.2	73.1	82.8
F2830	27.4	34.4	39.5	47.8	54.2	61.9	87.6	95.0	109.1	120.0	126.6	133.7
F2831	5.4	7.9	9.4	11.0	12.8	15.3	23.2	26.5	34.0	38.7	42.1	47.3
F2832	1.8	3.6	5.1	6.0	9.4	10.4	13.6	15.3	18.7	20.7	21.7	21.7
F2833	5.9	6.6	7.3	10.7	11.8	13.2	19.0	21.5	26.6	30.2	33.4	33.9
F2834	8.2	8.8	9.8	12.0	17.3	22.2	29.1	31.7	36.6	39.1	40.9	43.0
F2835	12.1	13.5	15.0	17.9	22.4	26.6	36.0	39.0	43.0	44.3	47.5	45.7

measurements of soil phosphorus, and $a, b_1, b_2, \dots, b_n$ are parameters. Conformity to this model may be indicated as before by the probability of obtaining an F ratio for the regression as large as or larger than that obtained experimentally if the true regression is zero in the population from which the sample is drawn. The probabilities found for the multiple linear models may be compared directly with the probabilities found for the simple linear models, whereas the F ratios cannot.

The principal reason for determining the phosphorus extracted by the anion exchange resin during different lengths of time was to obtain data that would permit application of the more general linear model described in the preceding paragraph. Two different methods were used to obtain from these data the multiple measurements of soil phosphorus to employ in the general linear model. In the first, the phosphorus-extracted *vs.* time-of-extraction curves were segmented into four different time intervals, namely, 0 to

TABLE VI

Phosphorus extracted from premoistened samples of different soils by the anion-exchange-resin method of Amer <i>et al.</i>												
Soil No.	P extracted in ppm of air-dry soil during indicated length of time in hours											
	0.084	0.17	0.25	0.50	1	2	8	12	24	36	48	72
F2814	15.0	18.9	19.0	21.0	22.1	25.3	35.1	40.3	47.3	51.9	54.7	58.6
F2815	0.0	0.8	1.2	1.3	1.5	1.8	2.3	2.6	3.3	3.8	4.3	4.9
F2816	9.5	10.7	11.1	12.3	14.3	17.5	25.0	28.1	35.6	41.3	46.0	54.3
F2817	10.0	10.6	11.1	14.3	17.5	24.2	34.1	36.9	43.1	45.3	48.0	52.2
F2818	10.6	14.7	16.0	18.5	22.0	27.5	35.6	37.8	41.0	41.7	41.9	41.9
F2819	1.9	2.5	2.8	3.0	3.8	4.7	7.4	8.9	11.2	12.7	14.0	15.5
F2820	0.0	0.0	0.0	0.6	3.5	5.1	9.3	11.3	14.1	15.5	16.3	17.1
F2821	4.5	4.9	5.3	6.0	6.8	8.4	12.4	14.0	17.4	20.1	22.1	23.6
F2822	0.8	1.2	1.6	2.0	2.3	3.3	6.2	7.1	8.9	9.2	9.2	9.2
F2823	0.0	0.7	1.0	1.3	2.0	2.6	2.6	2.6	2.6	2.6	2.6	2.6
F2824	8.9	9.2	9.7	10.2	12.1	13.8	20.1	23.0	28.3	32.0	34.9	38.6
F2825	16.7	19.9	22.4	29.2	34.4	40.6	50.3	53.7	60.7	65.6	69.6	76.8
F2826	1.2	2.0	2.2	2.5	2.8	3.1	4.1	4.4	4.8	5.0	5.0	5.0
F2827	3.1	3.4	3.7	4.1	4.8	5.7	9.2	10.7	13.3	14.8	15.7	17.3
F2828	7.8	9.3	11.3	13.0	14.9	16.2	22.8	24.4	26.5	27.3	27.5	27.5
F2829	25.8	27.3	27.5	29.2	31.3	34.7	45.9	50.5	60.6	68.3	75.2	88.1
F2830	39.5	41.0	43.3	45.1	49.3	54.1	78.7	86.3	101.4	112.2	121.0	136.9
F2831	6.0	6.3	7.8	8.4	10.2	12.2	19.7	22.8	28.5	32.4	35.7	40.3
F2832	1.4	1.9	2.9	3.4	4.1	5.4	7.9	9.3	12.6	15.0	17.1	20.9
F2833	6.3	6.5	6.8	7.5	8.0	9.5	13.2	14.5	17.6	19.4	21.0	23.2
F2834	12.6	14.1	18.2	19.2	19.7	20.1	23.3	24.9	28.4	31.3	33.3	35.0
F2835	7.4	10.7	12.7	14.3	16.7	22.6	32.0	35.4	42.0	46.5	50.3	56.7

0.25 hour, 0.25 or 2 hours, 2 to 24 hours, and 24 to 72 hours. The quantities of phosphorus extracted during these time intervals were taken as the independent variables, x_1 , x_2 , x_3 , and x_4 , respectively. The idea behind this method of obtaining different quantities of phosphorus is that the availability of a given atom of soil phosphorus to plants might be associated with the time at which it is sorbed by the resin. Since the resin rapidly sorbs phosphorus present in solution, most of the phosphorus sorbed during any time interval is released from the solid phase during that same time interval. Thus the phosphorus sorbed by the resin during the 0 to 0.25-hour interval might have greater availability than an equivalent amount sorbed during the 24 to 72-hour interval because of more rapid release from the solid phase on removal of the phosphorus from solution or more probably from a higher concentration of phosphorus in solution. The time intervals employed were chosen on the basis that, with a number of soils, changes in rate of phosphorus extraction

appeared to occur at approximately the beginning or end of the intervals chosen. When the data were treated in this fashion, the probabilities were found to be 8.9×10^{-4} and 2.6×10^{-5} for the initially dry, and premoistened samples, respectively. One of these values is higher and the other is about the same as those found with the simple linear model. The more complex method of analysis thus is less desirable than the simple method from the standpoint of both the probability and the time required to do the work.

The second method for obtaining the different phosphorus measurements for the general linear model was the one described by Amer *et al.*¹ In this method the phosphorus-extracted - time curve is represented as the sum of a group of simultaneous first-order reactions differing in rate. Amer *et al.* found that the curves they examined could be represented as the sum of three simultaneous reactions. In the present investigation some soils were found to have curves that could be broken down into four simultaneous reactions. Thus in the present work the general equation of the extraction curve may be represented by

$$P_r = P_\infty - (C_3 \times 10^{-k_3 t} + C_2 \times 10^{-k_2 t} + C_1 \times 10^{-k_1 t} + C_0 \times 10^{-k_0 t})$$

where P_r is the amount of phosphorus sorbed by the resin in time t , and P_∞ is the limiting amount of phosphorus sorbed by the resin as t approaches infinity. C_3 , C_2 , C_1 , and C_0 refer to the maximum quantities of phosphorus that can be sorbed by the resin in the slow, first intermediate, second intermediate, and fast reactions, respectively. The symbols k_3 , k_2 , k_1 , and k_0 give a measure of the rates of the slow, first intermediate, second intermediate, and fast reactions, respectively.

The parameters of the foregoing equation for the individual soils are listed in Table VII. Values of P_r , P_∞ , C_3 , C_2 , C_1 , and C_0 are in parts of phosphorus per million of air-dry soil. Values of k are derived on the basis of t in hours. Values of P_r calculated using these parameters agree closely with the observed values (except for a few soils during short time intervals in which no phosphorus was sorbed by the resin), thus indicating that a satisfactory fit has been obtained. Some of the curves can be represented by four simultaneous reactions, some by three, some by two, and some by one. Where the number of simultaneous reactions is less than four, the parameters are classified in Table VII in such a way that the rates

correspond as nearly as possible to those obtained with the soils having four reactions, and the value of C for the "missing" reactions is given as zero.

TABLE VII

Parameters in equations for representing the amount of inorganic phosphorus sorbed by anion-exchange resin from soils as a function of time of contact									
Sample No.	Numerical value of indicated parameters								
	C_3	$-k_3$	C_2	$-k_2$	C_1	$-k_1$	C_0	$-k_0$	P_∞
Premoistened samples									
F2814	30.9	0.0133	11.2	0.0775	19.9	5.75	0	—	62
F2815	4.2	0.0092	1.6	1.42	0	—	0	—	5.8
F2816	54.3	0.00613	10.0	0.215	9.7	13.6	0	—	74
F2817	33.5	0.00382	17.6	0.0678	10.2	0.408	8.7	26	70
F2818	14.3	0.0434	14.6	0.317	13.2	6.72	0	—	42.1
F2819	11.2	0.011	3.2	0.07	2.0	2.51	1.1	18.5	17.4
F2820	11.2	0.0238	3.0	0.087	2.9	0.531	0	—	17.1
F2821	18.1	0.020	1.9	1.11	4.3	16.7	0	—	24.2
F2822	7.2	0.0474	0.8	0.383	1.2	4.46	0	—	9.2
F2823	2.6	0.60	0	—	0	—	0	—	2.6
F2824	26.8	0.0115	5.6	0.112	1.6	0.787	8.5	17.7	42.5
F2825	45.7	0.00742	22.4	0.317	9.3	1.74	12.6	16.3	90
F2826	2.5	0.050	1.9	2.40	0.6	10.0	0	—	5.0
F2827	11.1	0.00516	7.0	0.0525	0.5	0.49	3.4	8.42	22
F2828	10.4	0.0437	5.6	0.203	11.5	4.11	0	—	27.5
F2829	118	0.00300	14.8	0.100	3.5	3.45	23.7	25.5	160
F2830	114	0.0046	34.7	0.087	3.8	2.06	37.5	29.4	190
F2831	32.2	0.00725	10.7	0.089	3.4	4.88	3.7	25.4	50
F2832	34.7	0.00299	3.0	0.0487	1.9	0.372	2.4	3.08	42
F2833	15.9	0.008	5.0	0.12	6.6	14.1	0	—	27.4
F2834	17.8	0.0139	4.1	2.25	15.1	6.24	0	—	37.0
F2835	44.4	0.00533	13.3	0.0854	6.2	0.256	11.1	5.00	75
Initially dry samples									
F2814	38.5	0.0156	9.9	0.884	16.2	3.72	0	—	64.6
F2815	8.7	0.0125	10.0	0.0464	1.4	2.24	0	—	20.1
F2816	40.3	0.0088	22.9	0.075	4.4	3.09	9.9	24.5	77.5
F2817	23.8	0.0159	7.6	0.101	7.5	0.92	6.2	1.58	45.1
F2818	13.5	0.0245	20.4	0.43	14.7	6.08	0	—	46.6
F2919	11.2	0.038	0	—	0	—	0	—	11.2
F2820	5.8	0.020	11.0	0.356	1.5	1.15	0	—	18.3
F2821	24.5	0.0197	4.6	0.124	1.0	16.0	0	—	30.1
F2822	12.6	0.021	1.1	0.60	0	—	0	—	13.7
F2823	3.0	0.0179	0	—	0	—	0	—	3.0
F2824	12.2	0.0309	14.3	0.432	14.8	4.97	0	—	41.3
F2825	16.6	0.0352	26.3	0.38	17.1	9.00	0	—	60.0
F2826	1.9	0.0295	2.6	0.102	0	—	0	—	4.5
F2827	4.6	0.0071	4.7	0.095	8.4	0.77	0	—	17.7
F2828	5.9	0.021	7.1	0.3	10.4	2.03	0	—	23.4
F2829	60.	0.0044	9.1	0.072	23.4	0.345	17.5	4.76	110
F2830	66.8	0.016	31.6	0.18	26.6	3.17	13.5	14.3	138.5
F2831	38.0	0.009	8.5	0.14	9.4	3.72	0.3	11.9	56.2
F2832	13.8	0.0222	5.3	0.362	3.9	3.16	0	—	23.0
F2833	24.2	0.0137	4.0	0.145	9.8	2.73	0	—	38.0
F2834	16.8	0.013	7.5	0.06	13.3	0.30	7.4	24.9	45
F2835	23.4	0.043	12.3	0.727	10.2	27.3	0	—	45.9

In the statistical analysis of the data the C values were separated into four categories according to the associated k values. The groups employed were $k = 0$ to -0.050 , $k = -0.051$ to -0.50 , $k = -0.51$ to -5.0 , and $k = -5.0$ to -29 . When this was done there were several instances in which two of the C values for a given soil had k values that fell in the same range. In these instances the two C values were added to give a single entry. After computing the regression of yield of phosphorus on C_3 , C_2 , C_1 , and C_0 , an analysis of variance was made and the F values were computed. The probabilities associated with the F ratios were found to be 3.1×10^{-4} and 6.7×10^{-4} for the initially dry, and premoistened samples, respectively. These probabilities are higher than those listed in Table VIII for the simple linear regression of yield of phosphorus on the phosphorus extracted by the resin method at

TABLE VIII

Probability of obtaining an F ratio as high as or higher than that found experimentally for the linear regression of the yield of phosphorus in sorghum plants in greenhouse cultures on the phosphorus measured in corresponding samples of soil by various laboratory methods		
Extraction method	Probabilities	
	Initially dry samples	Premoistened samples
0.01M CaCl_2 (Schofield-Aslyng)		
P concentration in solution		
20 g soil in 50 ml of solution	1.8×10^{-3}	1.7×10^{-3}
40 g soil in 50 ml of solution	2.1×10^{-3}	4.9×10^{-3}
Zero dilution	2.5×10^{-3}	3.9×10^{-3}
Phosphate potential	2.8×10^{-4}	9.5×10^{-4}
Antilog phosphate potential	1.7×10^{-3}	3.8×10^{-3}
0.1N HCl — 0.03N NH_4F (Bray and Kurtz)	1.0×10^{-1}	4.5×10^{-1}
0.5M NaHCO_3 (Olsen <i>et al.</i>)	1.3×10^{-4}	4.7×10^{-5}
Anion-exchange resin (Amer <i>et al.</i>)		
0.84-hour extraction	2.1×10^{-5}	2.6×10^{-5}
0.17-hour extraction	3.7×10^{-5}	1.9×10^{-5}
0.25-hour extraction	4.4×10^{-5}	2.6×10^{-5}
0.5-hour extraction	7.5×10^{-5}	5.3×10^{-5}
1-hour extraction	4.7×10^{-5}	8.2×10^{-5}
2-hour extraction	8.5×10^{-6}	1.0×10^{-4}
8-hour extraction	1.3×10^{-5}	9.2×10^{-5}
12-hour extraction	1.5×10^{-5}	6.7×10^{-5}
24-hour extraction	1.6×10^{-5}	3.3×10^{-5}
36-hour extraction	1.3×10^{-5}	1.6×10^{-5}
48-hour extraction	1.9×10^{-5}	1.0×10^{-5}
72-hour extraction	1.3×10^{-5}	4.2×10^{-6}

any of the individual time intervals. Thus from the standpoint of both the probability and the work involved the simple linear regression is superior to the multiple linear regression based on analysis of the curves expressing the amount of phosphorus sorbed by the resin versus the time.

An additional approach to the problem was made by combining into a single number the information obtained by extraction for different lengths of time and by calculating the simple linear regression of the yield of phosphorus on this number. The data were combined into a single number by summing the products of the rate of phosphorus sorption by the resin and the quantities sorbed during the various time intervals. Thus

$$\sum_i r_i q_i = \frac{q_1^2}{t_1} + \frac{(q_2 - q_1)^2}{t_2 - t_1} + \dots + \frac{(q_n - q_{n-1})^2}{t_n - t_{n-1}}$$

where r_i and q_i represent, respectively, the rate of phosphorus sorption and the quantity of phosphorus sorbed by the resin in the i 'th time interval, and where q_1 , q_2 , q_n , and q_{n-1} are the total amounts of phosphorus present on the resin at times t_1 , t_2 , t_{n-1} , and t_n , respectively. In practice, values of q were expressed in units of parts of phosphorus per million of soil and values of t were expressed in hours. Values of $\sum_i r_i q_i$ were found to be related

to the yield of phosphorus in a curvilinear form that could be fitted by the logarithmic expression, $\log(\text{yield of phosphorus}) = \text{constant} + [\text{constant'}] \times [\log(\sum_i r_i q_i)]$. The parameters in this equation

were found by the method of least squares for both the initially dry, and premoistened samples. The probabilities of obtaining an F value as large as or larger than those actually obtained if the true regression in the population is zero are 1×10^{-7} and 3×10^{-7} for the initially dry, and premoistened samples, respectively. These probabilities are lower than those obtained by the simple regressions using a fixed time interval (Table VIII) and indicate an improvement in prediction. Since the prediction was made on the basis of logarithms rather than the original numbers, however, the regression equations were used to compute the expected values of the numbers, and the regression of expected values on observed values was calculated. The probabilities associated with the F values for these regressions are 2.5×10^{-5} and 1.2×10^{-5} for the

initially dry, and premoistened samples, respectively. These values are similar to the ones in Table VIII, which indicates that the lower probabilities found with the logarithmic transformation can be explained on the basis of the selective alteration of numbers that occurs in the logarithmic transformation and hence that the method as such is not necessarily better than the much simpler approach of analyzing the resin for phosphorus after a single arbitrary time interval.

In view of the evidence that plants absorb the H_2PO_4^- ion preferentially over the HPO_4^{--} ion, some additional calculations were made to find whether any improvement in prediction could be made by taking this behavior into account. The concentrations of H_2PO_4^- and HPO_4^{--} in the 0.01M CaCl_2 extracts were calculated from the total-phosphorus concentration as described by Aslyng². The quantities of H_2PO_4^- and HPO_4^{--} sorbed by the anion exchange resin during a 2-hour contact period were calculated as described by Aslyng on the assumption that the proportions of the two ionic forms are determined by the pH value of the soil. Although the phosphorus may not be present in the resin in the indicated forms, it may be visualized that because the resin has essentially no effect on soil pH the calculated amounts of H_2PO_4^- and HPO_4^{--} represent the summation of the quantities of these forms of phosphorus present in solution at one time or another during the process of sorption of phosphorus from solution by the resin and release of phosphorus from the soil solid phase to the solution. The various conditions of pH measurement are indicated in Table IX.

A preliminary test of the significance of the H_2PO_4^- and HPO_4^{--} ion species was made by means of the model $y = a + b_1(\text{H}_2\text{PO}_4^-) + b_2(\text{HPO}_4^{--})$ where y is the yield of phosphorus in the plants, $(\text{H}_2\text{PO}_4^-)$ and (HPO_4^{--}) are the concentrations in the CaCl_2 extracts or the quantities of phosphorus sorbed by the resin in the respective forms, and a , b_1 , and b_2 are constants. Statistical tests showed that the regression coefficient b_2 did not differ significantly from zero in any case, and that the regression coefficient b_1 was highly significant in every case. These results indicate (a) that knowledge of the value of HPO_4^{--} did not lead to a significant increase in the precision of predicting the yield of phosphorus and (b) that the H_2PO_4^- ion was the important one in nutrition of the plants.

TABLE IX

Probability of obtaining an <i>F</i> ratio as high as or higher than that found experimentally for the linear regression of the yield of phosphorus in sorghum plants in greenhouse cultures on the H_2PO_4^- present in corresponding samples of soil		
Basis for calculating H_2PO_4^- in soil	Probabilities *	
	Initially dry samples	Premoistened samples
Micromolar concentration of P in 0.01M CaCl_2 extract		
20-g soil sample	1.7×10^{-3} (7.8×10^{-5})	3.9×10^{-4} (3.1×10^{-4})
40-g soil sample	1.7×10^{-3} (1.6×10^{-4})	1.7×10^{-3} (2.6×10^{-4})
Zero dilution	1.7×10^{-3} (2.8×10^{-4})	3.8×10^{-3} (9.5×10^{-4})
Micromolar concentration of P at zero dilution in 0.001M CaCl_2 (calculated from data obtained with 0.01M CaCl_2)	1.6×10^{-3}	3.7×10^{-3}
P extracted by anion exchange resin in 2 hours		
pH measured in 0.01M CaCl_2 , pK" calculated for 0.01M CaCl_2	1×10^{-7} (6.9×10^{-5})	3×10^{-7} (1.4×10^{-6})
pH measured in 0.01M CaCl_2 and calculated for 0.001M CaCl_2 , pK" calculated for 0.001M CaCl_2	1×10^{-7}	3×10^{-7}
pH measured in H_2O , pK" calculated for 0.001M CaCl_2	2×10^{-7}	7×10^{-7}

* Values in parentheses are based on the regression of the yield of phosphorus on the Briggsian logarithm of the concentration or quantity of H_2PO_4^- .

Because of the lack of statistical significance of the HPO_4^{--} ion, the values for HPO_4^{--} were neglected and the regressions were recalculated using H_2PO_4^- as the only independent variable. The probabilities associated with these regressions are shown in Table IX. The probability values associated with the 0.01M CaCl_2 extracts are somewhat lower where the regression calculations are based on H_2PO_4^- concentrations (Table IX) than on total-inorganic-phosphorus concentrations (Table VIII), indicating more precise prediction of the yields of phosphorus from H_2PO_4^- than from total inorganic phosphorus. Since the salt concentration and pH values in 0.01M CaCl_2 extracts are not those normal to the soils concerned, another set of pH values and H_2PO_4^- values was calculated for 0.001M CaCl_2 at zero dilution. The salt concentration in the nonsaline soils employed in this work is approximated more closely by 0.001M CaCl_2 than by 0.01M CaCl_2 . The probabilities

associated with the regressions are essentially the same with the 0.001*M* CaCl₂ as with the 0.01*M* CaCl₂, thus indicating no substantial improvement in prediction from making the calculations on the basis of the 0.001*M* concentration.

The results obtained with the anion-exchange resin are given in the lower part of Table IX. The probabilities are considerably below the corresponding values in Table VIII, indicating a marked improvement in prediction. Of all the methods tested in this paper, the most precise prediction of the yields of phosphorus is provided by the H₂PO₄⁻ sorbed by the anion-exchange resin. With the exchange resin there is no consistent difference in favor of the use of the pH values calculated for 0.001*M* CaCl₂. (The probability is slightly lower with the initially dry samples and slightly higher with the premoistened samples, but both differences are too small to affect the significant figures given in the tables employed for finding the probabilities).

Table IX includes some additional probability values that are derived from regressions of the yield of phosphorus on the logarithm of the H₂PO₄⁻ concentration or quantity instead of the original numbers representing the concentration or quantity. These values were computed to provide further insight into the phosphate potential.

From Tables VIII and IX it may be noted that the same probabilities are associated with the phosphate potential as with the logarithm of the concentration of H₂PO₄⁻ in the 0.01*M* CaCl₂ extract at zero dilution. Furthermore, the same probabilities are associated with the antilog of the phosphate potential as with the concentration of H₂PO₄⁻ in the CaCl₂ extract at zero dilution. The explanation is that (a) the phosphate potential differs from the logarithm of the H₂PO₄⁻ concentration by a constant factor and (b) the antilogarithm of the phosphate potential is equal to the product of the concentration of H₂PO₄⁻ and a constant factor. In both instances the constant factor includes the activity of calcium and the activity coefficient of H₂PO₄⁻. The probability is not changed if all the numbers in a series are increased or diminished by adding or subtracting a constant factor or if they are multiplied or divided by a constant factor. From this it may be concluded that the probabilities (and hence the value of the soil measurements in predicting the yield of phosphorus in the plants) are not affected

by transforming the H_2PO_4^- concentrations to antilogarithms of the phosphate potential by multiplying them by the activity coefficient of H_2PO_4^- and the activity of calcium or by transforming the logarithms of the H_2PO_4^- concentrations to phosphate potentials by adding the logarithms of the activity coefficient of H_2PO_4^- and the activity of calcium.

From the lower probability connected with the H_2PO_4^- concentration than with the total-phosphorus concentration and from the preceding discussion about the relative importance of H_2PO_4^- and HPO_4^{--} , it may be concluded that part of the superiority of the phosphate potential over the concentrations of phosphorus in the CaCl_2 extracts results from the fact that the phosphate potential is derived from the concentration of H_2PO_4^- , omitting the HPO_4^{--} . From the fact that the probabilities are lower with the logarithms of the concentrations of H_2PO_4^- than with the actual concentrations, it may be concluded that part of the superiority of the phosphate potential over the concentrations of phosphorus in the CaCl_2 extracts results from the selective alteration of numerical values that is a consequence of the logarithmic transformation. This improvement may be a result of the particular assemblage of data dealt with in the present investigation. In other trials the logarithmic transformation could conceivably have the opposite effect.

From the fact that the probabilities calculated for zero dilution are higher than those calculated for the original extracts (20-g and 40-g samples of soil in 50 ml of $10^{-2}M$ CaCl_2), it is concluded that there is no advantage in the present investigation from the extrapolation to zero dilution. The poorer results obtained by extrapolation to zero dilution probably can be accounted for by the magnification of experimental errors of measurement in the process of extrapolation.

One additional point that is pertinent in connection with the measurement of pH values of soils may be inferred from a comparison of the probabilities obtained where the pH measurement was made in a water suspension of soil (last row of figures in Table IX) with those obtained where the pH measurement was made in a CaCl_2 suspension (second and third from last row of figures in Table IX). The probabilities associated with the regression of the yield of phosphorus on the H_2PO_4^- sorbed by the resin are higher where the pH was measured in water than in CaCl_2 ; hence the

yield of phosphorus is predicted less precisely where the H_2PO_4^- values are calculated from pH values measured in water suspension than in CaCl_2 suspension. In the absence of a means of estimating the extent to which pH values measured in water suspension are biased from junction-potential effects, the comparison of results just described provides an operational test by means of which the practical significance of such bias may be inferred. Whatever the cause, however, the results suggest that for the purpose of predicting the yield of phosphorus in plants from the H_2PO_4^- sorbed by the exchange resin, it is better to make the pH measurements in CaCl_2 suspension than in water suspension.

SUMMARY

The yield of phosphorus in sorghum plants grown on samples of 22 different soils from the United States was determined in the greenhouse. Analyses of variance of the regressions of these values on the measurements of soil phosphorus by various laboratory methods were calculated as an aid in evaluating the methods.

No general advantage was found from incubating the samples in moist condition for a week before analysis versus making the analysis on the initially-dry samples. In the order of decreasing precision of predicting the yields of phosphorus the methods are as follows: (1) anion-exchange resin method of Amer and coworkers, (2) 0.5M NaHCO_3 method of Olsen and coworkers, (3) phosphate potential method of Schofield and Aslyng, (4) phosphorus concentration in the 0.01M CaCl_2 extract of Schofield and Aslyng, and (5) 0.1N HCl, 0.03N NH_4F method of Bray and Kurtz.

After taking into account the additional variables, little or no improvement in precision of prediction was obtained when the phosphorus-sorbed *vs.* time curves for the anion-exchange-resin method were (a) divided into four segments on the basis of time, the quantities of phosphorus in the four segments being used as independent variables in a multiple-regression equation, or (b) broken down into a maximum of four components on the assumption that the overall curve represents the summation of a group of simultaneous first-order reactions, the quantities of phosphorus in the several components being used as independent variables in a multiple-regression equation.

The precision of prediction was improved by using as the soil-phosphorus measurement the sum of the products of the rate of phosphorus extraction by the anion-exchange-resin method and the quantities of phosphorus extracted within individual time intervals. A logarithmic expression was used to fit the relationship, however, and it appeared that the greater precision of prediction resulted from the logarithmic transformation rather than the superiority of the method as such.

The precision of prediction was improved also by using the H_2PO_4^- concentration instead of the total-inorganic-phosphorus concentration as the independent variable in the 0.01M CaCl_2 extracts of Schofield and Aslyng and by using the H_2PO_4^- instead of the total inorganic phosphorus sorbed by the anion-exchange resin. This modification made the anion-exchange-resin method considerably better than the others tested.

Received July 21, 1958

LITERATURE CITED

- 1 Amer, F., Bouldin, D. R., Black, C. A., and Duke, F. R., Characterization of soil phosphorus by anion exchange resin adsorption and P^{32} -equilibration. *Plant and Soil* **6**, 391-408 (1955).
- 2 Aslyng, H. C., The lime and phosphate potentials of soils; the solubility and availability of phosphates. *Kgl. Vet. og Landbohøjskole Års.* **1954**, 1-50 (1954).
- 3 Bouyoucos, George John, A comparison between the suction method and the centrifuge method for determining the moisture equivalent of soils. *Soil Sci.* **40**, 165-171 (1935).
- 4 Bray, Roger H., and Kurtz, L. T., Determination of total, organic, and available forms of phosphorus in soils. *Soil Sci.* **59**, 39-45 (1945).
- 5 Dickman, S. R., and Bray, R. H., Colorimetric determination of phosphate. *Ind. Eng. Chem. Anal. Ed.* **12**, 665-668 (1940).
- 6 Fried, Maurice, and Dean, L. A., A concept concerning the measurement of available soil nutrients. *Soil Sci.* **73**, 263-271 (1952).
- 7 Olsen, Sterling R., Cole, C. V., Watanabe, Frank S., and Dean, L. A., Estimation of available phosphorus in soils by extraction with sodium bicarbonate. *U.S. Dept. Agr. Circ.* **939** (1954).
- 8 Schofield, R. K., The equilibrium concentration of phosphate in soil solutions. *In: Plant and Animal Nutrition in Relation to Soil and Climatic Factors. Proc. Specialist Conf. Agr. Australia 1949* (p. 124). H.M.S.O., London (1951).

THE EFFECT OF IRON SUPPLY ON THE YIELD AND COMPOSITION OF LEAVES OF TOMATO PLANTS

by E. S. TWYMAN

Botany Department, Rhodes University, Grahamstown, South Africa

INTRODUCTION

In an earlier paper (Twyman ⁷), it was suggested that the rate of iron supply had a profound effect on the iron metabolism of tomato and oat plants. The water-culture experiment described here was an attempt to investigate this effect. At the same time it was decided to obtain information concerning the distribution of nutrient elements in the tomato plants at the various iron treatments.

METHODS AND DESIGN OF THE EXPERIMENT

1. *Cultural procedure*

Tomato plants (var. Marglobe) were grown singly in litre flasks containing culture solutions based on Arnon's macronutrient and micronutrient solutions, slightly modified to conform with the design of the experiment. Iron was supplied by appropriate dilutions of a stock solution made from a mixture of ferrous sulphate (10.2 g per litre) and citric acid (14.1 g per litre). The iron treatments were started 42 days after sowing. Four levels of total iron supply were used, namely, $Fe_1 = 0.047$, $Fe_2 = 0.094$, $Fe_3 = 0.187$ and $Fe_4 = 0.375$ mg per litre, and each of these amounts was administered in three ways during a sixteen day cycle, thus:

- a) one full dose at the beginning of each cycle,
- b) half the full amount given twice at 0 and 8 days after the commencement of each cycle,
- c) a quarter of the full quantity given four times at 0, 4, 8 and 12 days after the beginning of each cycle.

All cultures received 0.25 mg Mn per litre at the beginning of each 16-day cycle and they were all maintained at the 1-litre mark by additions of

redistilled water. Each treatment was replicated four times, and three 16-day cycles were completed before cropping.

The aim of this design was to regulate the rate of iron supply at the four total-iron levels, and it was assumed that the administration of one dose supplies iron more rapidly than if supplied in two or four increments.

2. *Cropping of plants*

At the end of the experiment each plant was divided into root and shoot systems. The shoot system was further divided into three parts on the basis of the number of leaves per plant. The total number of expanded leaves borne by each plant was divided by three and each third of the leaves and stem was dried and stored separately. Thus, as far as the leaves were concerned, these samples were designated lower leaves, middle leaves and upper leaves. The plant organs were dried at 100°C in a ventilated hot-air oven, crushed, weighed and finally ground to a fine powder in an agate ball mill.

3. *Chemical analysis*

Prior to determining nitrogen, phosphorus, potassium, sodium, magnesium and calcium, 0.1 to 0.2 g of finely ground leaf tissue was digested with 2 ml concentrated sulphuric acid and small additions of hydrogen peroxide. When a clear digest was obtained it was quantitatively diluted to 100 ml and suitable aliquots were used for estimating the above-mentioned elements. In the cases of iron and manganese, 5 to 6 g of dried material were ashed in silica beakers in a muffle furnace at 500°C. The ash was taken up in 15% nitric acid digested on a hot plate for an hour, filtered and quantitatively made up to 100 ml. Aliquots of this solution were used for estimating iron and manganese.

The following statements summarise the analytical methods used:

a) Nitrogen was estimated colorimetrically using Jackson's modification of Nessler's reagent.

b) Phosphorus was determined colorimetrically by neutralising an aliquot of the digest and developing the molybdenum-blue complex with ammonium molybdate in sulphuric acid and stannous chloride.

c) Magnesium was estimated by the Titan Yellow method using a carefully standardised procedure.

d) Potassium and sodium were estimated by means of a flame photometer calibrated with artificial ash solutions in sulphuric acid of the same concentration as the test solutions.

e) Calcium was determined by titrating with disodium versenate using murexide as indicator.

f) Iron was determined by the thioglycollic acid method.

g) Manganese was estimated colorimetrically by the potassium periodate method.

RESULTS

1. Symptoms observed

The symptoms observed at harvest were those of iron deficiency and manganese toxicity. In severe cases of the latter, small brown spots or flecks occurred in the chlorotic areas. In general, at all four total-iron supplies the plants receiving their iron in one dose showed less severe iron chlorosis and/or manganese toxicity than the plants supplied with iron in two doses, while those receiving four doses were most severely affected.

At the Fe_4 treatment the plants receiving the iron in one dose were all normal green plants, those receiving the iron in two doses were slightly chlorotic, particularly the upper leaves, while those receiving the iron in four doses were more markedly chlorotic and had slight manganese toxicity. The plants receiving Fe_3 treatment in one dose showed slight chlorosis and brown flecking in the top leaves, however, when they received the iron in two doses these symptoms were more pronounced, and when the iron was administered in four increments the symptoms were even more severe. This trend in the occurrence of chlorosis and brown flecking was followed at the Fe_2 and Fe_1 treatments with increasing severity.

2. Total dry weight

An analysis of variance of the total dry weight yields shows that the two main effects (*i.e.* total iron supply and number of iron doses) are significant. The total-iron-supply effect is highly significant at the 0.1% level and the data in Table Ia show that as the iron supply is increased the mean dry weight per plant increases. The difference however between the mean yields at the Fe_3 and Fe_4 treatments is not significant.

The effect of the number of doses on the dry-weight yields is only significant at the 5% level and reference to Table Ib shows that only those plants receiving four doses of iron have their mean dry-weight yields significantly reduced below those receiving one or two doses of iron. Thus if it is assumed that the administration of iron in four doses supplies iron less rapidly to the plants than when it is supplied in one or two doses, then there is some indication that the slower the rate of supply of iron the lower the yield. This effect is presumably due to internal heavy-metal

TABLE I

The effect of total iron supply and number of iron doses on the mean dry-weight yield per plant in grams				
a	Total-iron supply			
	Fe ₁	Fe ₂	Fe ₃	Fe ₄
	4.50	6.88	9.80	11.00

S.E. of mean = ± 0.50				
b	No. of doses			
	1	2	4	
	8.58	8.60	6.95	

S.E. of mean = ± 0.43				

antagonisms which reduce the efficient utilisation of iron. This point will be discussed further later on in this paper.

3. Iron in the leaves

Statistical analysis of the concentration of iron in lower, middle and upper leaves at the various iron treatments shows that only two of the main effects (*i.e.* leaf position and total iron supply) are of significance (at 0.1% level).

Tables II, III and IV summarise the influence of the three main effects (total iron supply, leaf position and iron dosage) on the concentration of the various elements in the dry leaf tissue.

TABLE II

The mean concentrations of elements in the dry leaf tissue of tomato plants as influenced by the total-iron supplies						
Element conc.	Total-iron supply				S.E. of means	Significance
	Fe ₁	Fe ₂	Fe ₃	Fe ₄		
Fe ppm	112	125	129	144	± 3.9	S-0.1%
Mn ppm	219	168	122	105	± 6.4	S-0.1%
N %	2.57	2.42	2.38	2.58	± 0.074	NS
P %	0.84	0.67	0.56	0.57	± 0.016	S-0.1%
K %	4.83	4.75	4.80	4.73	± 0.104	NS
Na %	0.056	0.069	0.039	0.046	± 0.002	S-0.1%
Ca %	3.20	2.98	2.85	2.85	± 0.044	S-0.1%
Mg %	0.64	0.56	0.47	0.44	± 0.012	S-0.1%

S $\equiv$ Significant differences between means exist

NS $\equiv$ No significant differences

TABLE III

The mean concentrations of elements in the dry leaf tissue of tomato plants as influenced by the leaf position					
Element conc.	Leaf position			S.E. of means	Significance
	Lower	Middle	Upper		
Fe ppm	154	141	88	± 3.4	S-0.1%
Mn ppm	223	149	88	± 5.5	S-0.1%
N %	1.8	2.2	3.4	± 0.064	S-0.1%
P %	0.52	0.54	0.92	± 0.014	S-0.1%
K %	5.31	4.75	4.72	± 0.10	S-0.1%
Na %	0.060	0.053	0.044	± 0.002	S-0.1%
Ca %	4.54	2.92	1.45	± 0.038	S-0.1%
Mg %	0.58	0.53	0.47	± 0.014	S-0.1%

TABLE IV

The mean concentrations of elements in the dry leaf tissue of tomato plants as influenced by the number of iron doses					
Element conc.	No. of iron doses			S.E. of means	Significance
	One	Two	Four		
Fe ppm	131	129	123	± 3.4	NS
Mn ppm	134	156	171	± 5.5	S-1.0%
N %	2.57	2.45	2.44	± 0.064	NS
P %	0.61	0.65	0.73	± 0.014	S-0.1%
K %	4.64	4.82	4.88	± 0.10	NS
Na %	0.56	0.55	0.46	± 0.002	S-1.0%
Ca %	2.99	2.88	3.02	± 0.038	NS
Mg %	0.52	0.53	0.54	± 0.014	NS

The effect of the total iron supply (Table II) is such that irrespective of leaf position and dosage the mean iron concentration in the leaves from plants receiving the lowest iron supply is less than that obtained in the leaves from plants receiving 0.094 and 0.187 mg Fe per litre and the leaves from these treatments have a significantly lower iron concentration than those from plants supplied with the highest amount of iron. The leaf-position effect (Table III) is such that the lower leaves accumulate more iron than the middle leaves, which in turn contain more iron per unit of dry weight than the upper leaves. This distribution of iron is well known. The iron dosage (Table IV) and the three first-order interactions have no significant effect on the concentration of iron in the leaves.

4. Manganese in the leaves

The manganese concentration in the leaves is significantly affected by the total iron supply, leaf position and iron dosage (all at the 1% level). In addition there is a significant interaction between total-iron supply and leaf position at the 1% level.

The mean manganese concentration in the leaves diminishes as the total iron supply is increased from the Fe_1 to the Fe_3 level of iron supply (Table II). The apparent decrease in the manganese concentration from the Fe_3 to the Fe_4 treatment is not significant. As regards the leaf-position effect (Table III) it is evident that the lower leaves accumulate more manganese than the middle leaves, which in turn contain less than the upper ones. The number of doses of iron administered affects the manganese concentration in such a way that as the number of doses increases from one to four the concentration increases from 134 to 171 ppm (Table IV).

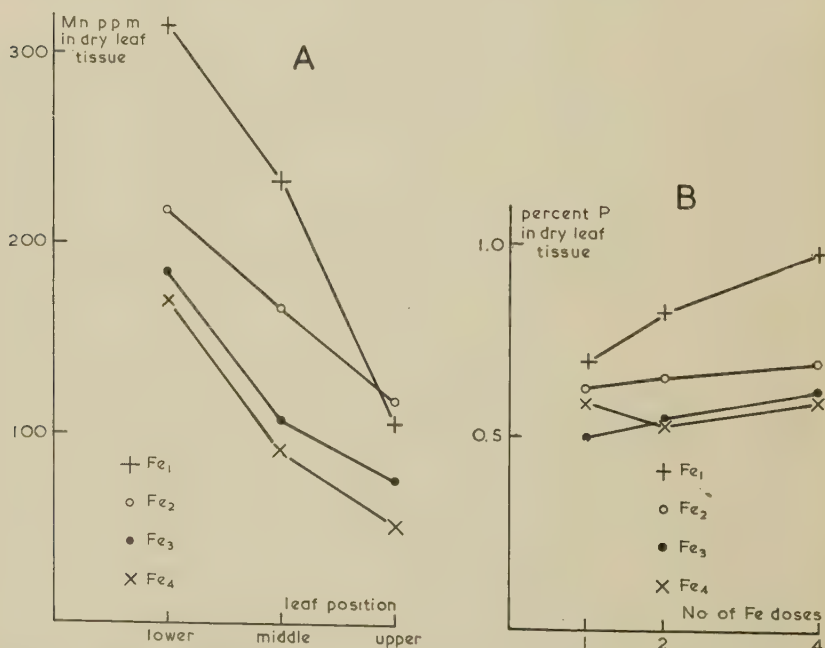


Fig. 1. Shows the relationship between (a) manganese concentrations (ppm) in the dry leaf tissues and the leaf position at the four iron supplies (S.E. of means = ± 11.0); (b) the percentage phosphorus concentration in the dry leaf tissue and number of doses of iron at the four total-iron supplies (S.E. of means = ± 0.027).

The effect of the first-order interaction, total iron supply $\times$ leaf position, is shown in Fig. 1a. At the Fe_1 level of iron supply the falling gradient of manganese concentration from the lower to the upper leaves is much steeper than that found at the Fe_2 , Fe_3 and Fe_4 iron treatments.

5. *Nitrogen in the leaves*

Only the leaf-position main effect is significant (1% level) and the expected trend is observed. The mean percentage nitrogen in the leaves decreases from the upper to the lower leaves (Table III). The total iron supply and iron dosage have no effect on the distribution of nitrogen.

6. *Phosphorus in the leaves*

The three main effects and the first-order interaction, total iron supply $\times$ iron dosage are all highly significant. As the total iron supply increased from Fe_1 to Fe_3 there is a significant fall in the mean phosphorus concentration in the leaves (Table II). There is no significant difference between the mean phosphorus content of leaves from plants receiving the Fe_3 and Fe_4 treatments. The leaf-position effect (Table III) is such that the upper leaves accumulate more phosphorus than the middle and lower leaves. As regards the number of iron doses (Table IV), it is evident that the leaves from plants receiving four increments of iron contain more phosphorus than those from plants receiving their iron in one or two doses. Thus the slower the rate of iron supply to the plants the higher the phosphorus content of the leaves.

The rate of change in phosphorus concentration in the leaves as the number of iron doses increased from one to four varies with the total iron supply (Fig. 1b). Thus at the lowest iron supply (Fe_1) the rising gradient of phosphorus concentration in the leaves with increasing number of doses is steeper than that at Fe_2 , Fe_3 and Fe_4 levels. In other words, the decreasing rate of iron supply at the lowest iron level results in a sharper rise in the phosphorus content than at the other levels of iron supply. This effect is significant at the 1% level.

7. *Potassium in the leaves*

The only significant main effect is that of leaf position and is

such that the lower leaves tend to accumulate more potassium than the upper ones (Table III). In addition to this there is a highly significant (0.1%) interaction between total iron supply and leaf position.

The total iron supply $\times$ leaf position interaction is clearly shown in Fig. 2a and b.

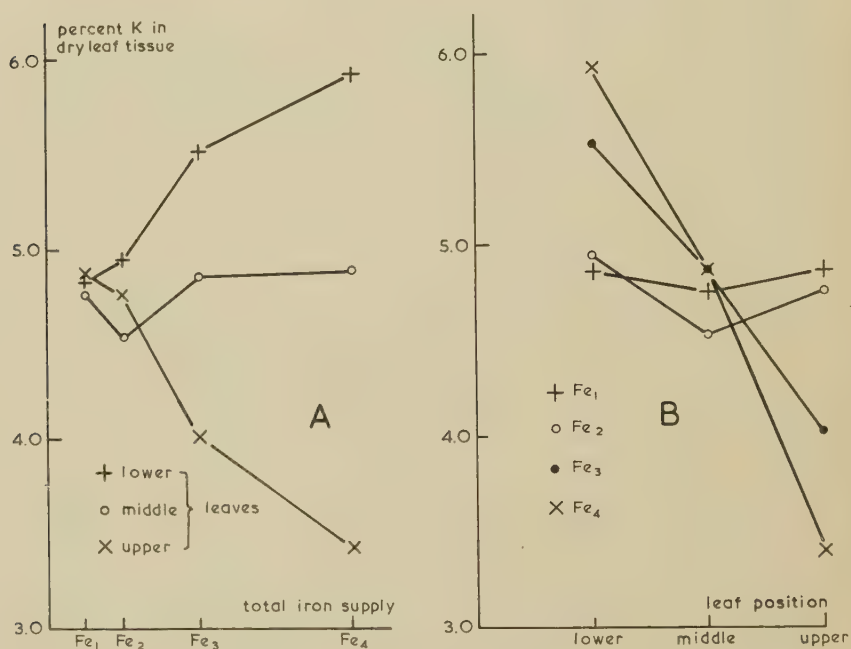


Fig. 2. Shows the relationship between the percentage potassium concentration in the dry leaf tissues and (a) the total iron supply, (b) the leaf position (S.E. of means = ± 0.21).

It is obvious from these figures that the change in potassium concentration with increasing iron supply is quite different at the three different leaf positions. With increasing total iron supply there is (1) a rise in the potassium concentration in the lower leaves, (2) little or no change in the potassium content of the middle leaves and (3) a marked fall in potassium concentration in the upper leaves (Fig. 2a). Furthermore the fall in potassium concentration from the lower to the upper leaves varies with the iron supply; this is such that the falling gradient is steepest at

the highest iron level (Fe_4), less steep at the Fe_3 level, and no significant change at the two lowest iron supplies (Fig. 2b).

8. Sodium in the leaves

Reference to Tables II, III and IV indicates that all three main effects are highly significant. As regards the total-iron-supply effect it appears that sodium tends to accumulate at the lower iron supplies (Fe_1 and Fe_2) as compared with the higher levels (Fe_3 and Fe_4). With respect to leaf position, sodium tends to accumulate in the lower leaves to a greater extent than in the middle leaves, while the upper leaves have the lowest percentage sodium. Iron administered in one or two doses has no significant effect on the sodium content of the leaves, but when supplied in four doses a significant fall in sodium content is observed. No significant interaction was found.

9. Calcium in the leaves

The calcium content of the leaves is affected significantly by the total iron supply and by the position of the leaves. Both these main effects are significant at the 0.1% level. In Table II it is seen that as the iron supply increases the calcium content in the leaves falls, and from Table III it is observed that the lower leaves accumulate calcium to a much greater extent than the middle leaves, while the upper leaves have the lowest mean concentration.

Two of the first-order interactions, namely, total iron supply $\times$ leaf position, and total iron supply $\times$ iron dosage, are highly significant at the 0.1% level (Table V).

TABLE V

The total iron supply $\times$ leaf position and the total iron supply $\times$ iron dosage interaction tables. Mean percentages of calcium in the dry leaf tissue S.E. of means = ± 0.076				
Factor	Total iron supply			
	Fe_1	Fe_2	Fe_3	Fe_4
<i>Leaf position</i>				
Lower	4.65	4.34	4.36	4.80
Middle	3.41	3.03	2.75	2.49
Upper	1.54	1.57	1.43	1.25
<i>No. of doses</i>				
1	3.63	2.79	2.73	2.83
2	2.91	2.92	2.73	3.00
4	3.07	3.23	3.07	2.71

With increasing iron supply the changes in calcium content of the leaves are as follows: (1) in the lower leaves there is an initial fall followed by a gradual rise; (2) in the middle leaves there is a very pronounced continuous fall in calcium level; (3) in the upper leaves there is a tendency for the concentration to fall slightly. It will be seen (Table V) that the changes in calcium content of the leaves with respect to iron dosage and total iron supply are erratic, and the effect needs further investigation.

10. Magnesium in the leaves

The position of leaves and total iron supply have highly significant (0.1% level) effects on the distribution and mean concentration of magnesium in the leaves. As regards the iron supply (Table II), the leaves from plants receiving the higher iron levels

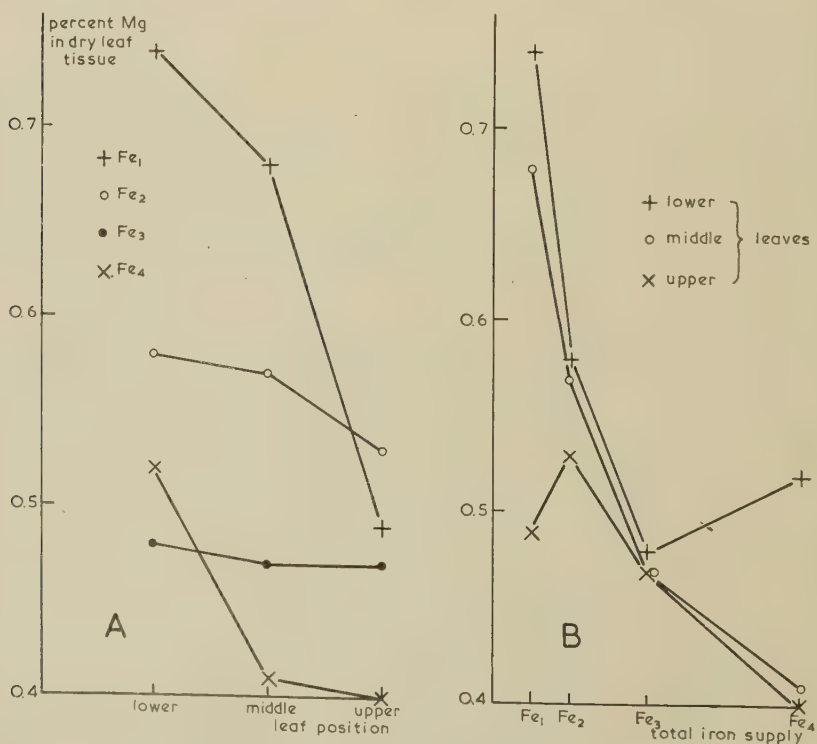


Fig. 3. Shows the relationship between the percentage magnesium concentration in the dry leaf tissues and (a) the leaf position and (b) the total iron supply (S.E. of means = ± 0.027).

have a lower magnesium content than those from plants receiving the low iron supplies. From Table III it is observed that the lower leaves accumulate more magnesium than the upper ones. There is also a possible first-order interaction between the leaf position and total iron supply, significant only at the 5.0% level.

The magnitude of the total iron supply appears to affect the steepness of the fall in magnesium content from the lower to the upper leaves (Fig. 3). The falling gradient of magnesium concentration is steeper at the lowest iron level than at the other three (Fig. 3a). Furthermore, with increasing iron supply the rate of fall in magnesium content in the lower and middle leaves is greater than in the upper ones (Fig. 3b). However, since these apparent effects are only significant at the 5% level, it is suggested that further investigation is required.

DISCUSSION

Throughout this discussion it is assumed that the administration of any particular total iron supply in one dose every sixteen days supplies iron to the plant more rapidly than if given in two half doses (*i.e.* once every 8 days), and that this latter treatment supplies iron more rapidly than when the iron is administered in four quarter doses (*i.e.* once every 4 days). It is realised that this is an over-simplification of the actual conditions existing in the cultures, but having regard to the design of the experiment it is a fair approximation to make.

The rate of iron supply had a marked effect on the occurrence of iron-deficiency chlorosis. The slower the rate of iron supply the more pronounced was the chlorosis; this was particularly evident at the Fe_3 and Fe_4 iron levels. The yield data are correlated with these observations since the overall effect of the rate of iron supply was to depress the total dry-weight yield at the slowest rate of supply (Table Ib). Contributory factors responsible for the development of chlorosis are (1) the increased manganese concentration in the leaves (Table IV), and (2) the increased phosphorus concentration in the leaves (Table IV) as the rate of iron supply is reduced. In this connection also it should be noted that the iron content of the leaves is not affected by the rate of iron administration (Table IV). These observations suggest that under conditions

of slow entry of iron into the plant, manganese succeeds in preventing the normal reactions leading up to the formation of chlorophyll from taking place. The mechanisms by which this may occur have been suggested by Granwick³, Theorell⁶ and Gjessing and Sumner.² Hewitt⁴ has also suggested that manganese may operate in this way in spinach. The enhanced phosphorus content of the leaves as the rate of iron entry is reduced may favour the formation of inactive iron phosphates (Somers and Shive⁵), thus aggravating the chlorotic condition. This chlorosis characteristic of iron deficiency is not due to a shortage of iron since (1) the lowest recorded iron concentration in this experiment was 75 ppm and (2) the leaves of many of the chlorotic plants possessed iron concentrations well in excess of this (*e.g.* 111 ppm). It is evident that the iron present in the plant is in some stable inactive state and that the rate of iron entry into the plant is a factor concerned with this inactivation. It remains to be ascertained precisely what "inactive" compounds are formed with iron in these chlorotic plants possessing apparently adequate iron.

The total-iron level irrespective of dosage (or rate of supply) had significant effects on (1) the yield (Table Ia), (2) the concentrations of iron, manganese, phosphorus, sodium, calcium and magnesium in the leaves (Table II). There was no significant change in the nitrogen and potassium concentration in the leaves. The general effect of increasing the iron level was to decrease the concentration of manganese, phosphorus, sodium, calcium and magnesium in the leaves, while the iron concentration was increased. The change in concentration of all the elements investigated in the leaves with respect to leaf position were all significant (Table III). Iron, manganese, potassium, sodium, calcium and magnesium all decreased, while nitrogen and phosphorus increased in concentration from the lower to the upper leaves. These observations indicate how the supply of one nutrient element affects the analysis of the leaf for other elements, and underline the importance of the age of a leaf sample utilised in diagnosing nutrient status.

The first-order interaction total iron supply $\times$ leaf position was significant in the case of manganese, potassium, calcium and magnesium concentrations in the leaves. The trends in manganese and magnesium concentration at the three leaf positions with increasing

iron supply show a marked similarity, since in both cases the rate of fall in concentration is greater in the lower and middle leaves than in the upper leaves (Figs. 1a and 3a). The trends in calcium concentration with respect to this interaction (Table V) are similar in that the falling gradient with increasing iron supply in the middle leaves is steeper than in the upper leaves. The potassium concentration in the leaves as affected by the interaction is shown in Fig. 2 and the trends are similar to those obtained by Bolle-Jones¹ in potato. He observed that increasing the iron supply decreased the soluble potassium concentration of the young laminae of potato, but increased the potassium concentration of the old laminae. In this experiment increasing the iron supply decreased the potassium content of the upper or younger leaves and increased the potassium content of the lower or older leaves, while the potassium concentration in the middle leaves is unaffected (Fig. 2).

The iron dosage $\times$ total iron supply interaction was significant only in respect to the phosphorus and calcium concentration in the leaves. It is of interest to note that at the lowest iron level (Fe_1) decreasing the rate of iron supply brings about a marked increase in the phosphorus concentration in the leaves (Fig. 1b). The effect of this interaction on the calcium concentration is obscure and needs further elucidation.

The results of this experiment have shown that irrespective of the total amount of iron "available", the rate of supply over a given period of time is a factor influencing the occurrence of chlorosis in tomato plants. Increasing the total iron supply from 0.047 to 0.375 mg per litre brings about (1) an increase in the yield and the iron concentration in the leaves, and (2) a decrease in the manganese, phosphorus, sodium, calcium and magnesium concentration in the leaves. The concentration of iron, manganese, potassium, sodium, calcium and magnesium in the leaves increases from the young to old leaves, while the concentration of nitrogen and phosphorus decreases. A slow rate of iron supply decreases the yield and the sodium content of the leaves while the manganese and phosphorus concentration in the leaves increases. These results are of interest in connection with iron metabolism and in evaluating the results of leaf analysis designed to assess nutrient status of plants.

SUMMARY

This paper describes a factorial experiment which was designed to elucidate the effect of total iron supply and rate of iron supply on the yield of tomato plants. Information was also obtained on the effect of iron supply and leaf age on the concentration of a number of nutrient elements in the leaf tissue.

Increasing the total iron supply increases the yield, and the iron concentration in the leaves, while the manganese, phosphorus, sodium, calcium and magnesium decrease in concentration. The young leaves have a lower concentration of iron, manganese, potassium, sodium, calcium and magnesium than the older leaves, while the reverse is true of nitrogen and phosphorus. A slow rate of iron supply decreases the yield and sodium concentration in the leaves but increases the manganese and phosphorus concentrations.

Interaction between total iron supply and leaf position affects the manganese, potassium, calcium and magnesium concentrations in the leaves, while the interaction between total-iron level and rate of iron supply affects the phosphorus and calcium concentration in the leaves.

ACKNOWLEDGEMENTS

The author wishes to acknowledge his indebtedness to the South African Council for Scientific and Industrial Research for providing the salary of a technical assistant and a grant towards the running expenses incurred during the progress of this work. The author is also grateful for the assistance rendered by Miss S. Gillespie, who carried out the routine analytical and cultural work in connection with this investigation.

Received May 22, 1958

LITERATURE CITED

- 1 Bolle-Jones, E. W., The interrelationships of iron and potassium in the potato plant. *Plant and Soil* **6**, 129 (1955).
- 2 Gjessing, E. C. and Sumner, J. B., Synthetic peroxidases. *Arch. Biochem.* **1**, 1-18 (1943).
- 3 Granwick, S., Protoporphyrin 9 as a precursor of chlorophyll. *J. Biol. Chem.* **172**, 717-727 (1948).
- 4 Hewitt, E. J., Metal interrelationships in plant nutrition 2. The relation of metal toxicity, molybdenum and nitrogen source to chlorophyll and magnesium content of beet in sand culture. *J. Exptl. Botany*, **5**, 110-118 (1954).
- 5 Somers, I. I. and Shive, J. W., The iron-manganese relation in plant metabolism. *Plant Physiol.* **17**, 582 (1942).
- 6 Theorell, H., Heme-linked groups and mode of action of some heme-proteins. *Advances in Enzymol.* **7**, 265-303 (1947).
- 7 Twyman, E. S., The iron and manganese requirements of plants. *New Phytologist* **50**, 210-226 (1951).

THE GROWTH AND COMPOSITION OF NATURAL STANDS OF BIRCH

2. THE UPTAKE OF MINERAL NUTRIENTS

by J. D. OVINGTON * and H. A. I. MADGWICK

The Nature Conservancy, Merlewood Research Station, Grange-over-Sands, Lancashire

INTRODUCTION

Data of the dry weights and production of dry matter for individual trees and natural stands of birch growing on fen peat have been published in a previous paper (Ovington and Madgwick ⁹). The birch woodlands form a series ranging in age from 6 to 55 years and by analysing the sample trees of *Betula verrucosa* it has been possible to determine the incorporation of certain chemical elements within the tree stands. The amounts of sodium, potassium, calcium, magnesium, phosphorus and nitrogen in trees of differing ages have been calculated and estimates made of the gross and annual nutrient uptake by the plantations.

METHODS

Two birch trees of approximately average size were felled in each of the nine woodlands. Six additional trees, giving a representative range of sizes for the stand, were sampled in the youngest of the woodlands in order to determine the variation between trees within the plot. A large sample of the leaves and branches as well as samples of the boles, taken at two-metre intervals along the tree trunks, were collected from each tree and oven dried at 80°C. When dry, the samples were ground in a Christy and Norris mill to pass through a sieve with round holes of 0.4 mm diameter and then stored for chemical analysis.

Nitrogen was determined by the micro-Kjeldahl method. To determine the other elements the wet-ashing procedure, as described by Piper ¹⁰, was followed using nitric, perchloric and sulphuric acids. The sodium and po-

* Now at the Department of Botany, Minneapolis, Minnesota, U.S.A.

tassium contents of the resultant acid solution were measured with a flame photometer, calcium by the versenate technique, magnesium by the titan yellow and phosphorus by the molybdate-blue methods. The results obtained were combined with the dry weight data to estimate the amounts of the various elements in the trees and tree stands.

The peat soils in four plots were sampled by taking five cores of 10 cm diameter to a depth of 50 cm in each plot. The soil samples were dried and analysed as for the tree samples.

CHEMICAL COMPOSITION AS A PERCENTAGE OF THE DRY WEIGHT

Some variation in chemical composition occurs within the leaves, branches, bole and roots of individual trees. The percentage values given in Fig. 1 have been calculated to be representative of the

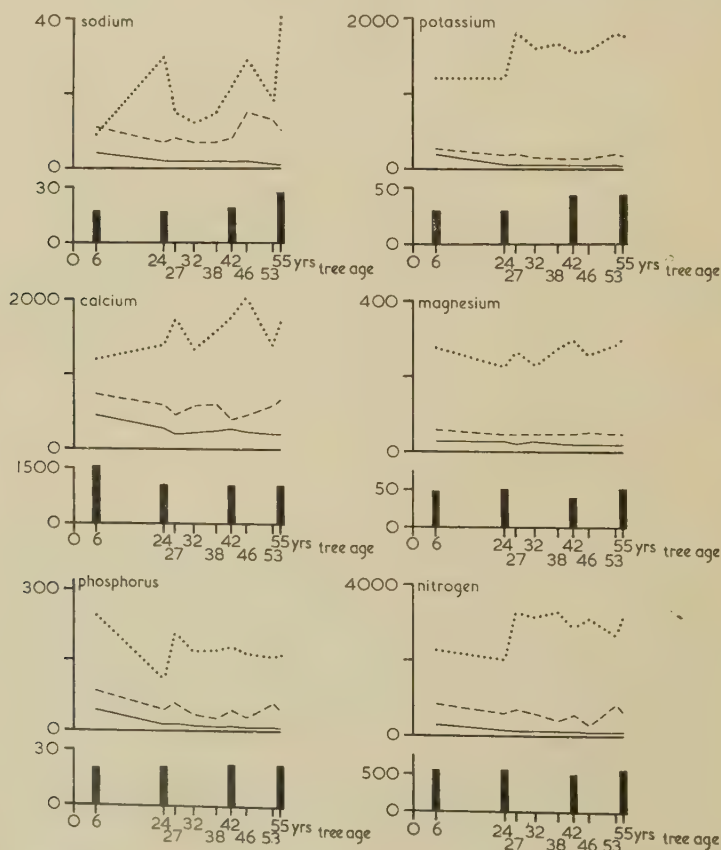


Fig. 1. The composition (mg/100 g O.D.) of the peat soil (histograms) and birch trees (leaves, live branches -----, bole ———).

whole of each respective part of the trees. The average percentages of all six elements decrease in the order leaves, branches and bole with the roots being broadly comparable in nutrient content to the branch or bole material. Nitrogen, potassium and calcium together represent about 93 per cent of the total weight of all six elements. The relative proportions of the various elements differ considerably for different parts of the trees, for example, the percentage of nitrogen in the leaves is almost double that of potassium or calcium whilst in the boles the percentage of calcium is nearly twice that of nitrogen and four times that of potassium (Table I).

TABLE I

The average nutrient content of sample trees as percentages of the oven-dry weight				
Nutrient	Leaves	Branches	Boles	Roots
Sodium	0.02	0.01	0.002	0.01
Potassium	1.56	0.18	0.07	0.09
Calcium	1.57	0.56	0.25	0.35
Magnesium	0.27	0.05	0.02	0.03
Phosphorus	0.17	0.05	0.01	0.02
Nitrogen	2.85	0.60	0.14	0.41

The average percentages of nutrients in the leaves, branches, bole or roots vary considerably for different sample trees but frequently there appears to be some relationship to tree age or height. For instance, the leaves of the trees from the older plots generally have greater percentages of sodium, potassium, calcium, magnesium and nitrogen but smaller values of phosphorus than the leaves of the younger sample trees. The average concentrations of all six elements tend to be smaller in the older boles but for the living branches there is an initial period of decreasing concentration of nutrients in the juvenile stages followed by an increase in the more mature trees.

Although soils from only four of the nine plots were sampled, it seems clear that the differences in the percentages of the elements for sample trees cannot be attributed to differences in the soil conditions of the plots. The decreasing concentration of nutrients within the tree boles is almost certainly related to the different chemical compositions of bark, sapwood and heartwood and to the change with age in the proportions of these in the boles (Wright and Will¹⁶). Comparable changes have been recorded up individual trunks, thus samples from the young distal two metres of bole con-

sistently contain higher concentrations of nutrients than samples from the older and lower part of the stem with a larger proportion of heartwood. The initial decrease in the percentages of the elements in the branches may result from the increasing average size of the branches as the trees age. The differences in the nutrient content of the leaves from the various trees are probably caused by a variety of factors. Leyton⁴ has demonstrated that differences occur in the nutrient content of spruce, pine or larch needles depending on tree size within a stand. Comparisons of the leaves of the eight trees representing the size range in the younger plot have shown that the concentrations of potassium, phosphorus and nitrogen in the leaves are negatively correlated with tree height whilst sodium, calcium and magnesium are positively correlated (Table II), but for sodium and nitrogen the correlation is not significant at the 5 per cent level. Since the less vigorous trees in a plot are likely to be suppressed and die, the average trees are being selected from a progressively smaller group of faster growing trees and this could cause a gradual change in average leaf composition. However, this does not appear to have any significant effect on the change of leaf composition as the stands mature, for potassium decreases with height within a plot but increases with height between plots.

TABLE II

Correlation coefficients between tree height and percentage composition						
Nutrient	For the average trees from the nine plots			For the eight sample trees from the 6 yr. old plot		
	Leaves	Branches	Boles	Leaves	Branches	Boles
Sodium	+0.594	+0.439	—0.866 **	+0.477	—0.550	—0.532
Potassium	+0.731 *	—0.637	—0.809 **	—0.730 *	—0.171	—0.073
Calcium	+0.701 *	—0.446	—0.818 **	+0.752 *	—0.809 *	—0.601
Magnesium	+0.333	—0.461	—0.883 **	+0.922 **	—0.274	—0.678
Phosphorus	—0.446	—0.525	—0.826 **	—0.727 *	—0.316	—0.548
Nitrogen	+0.526	—0.378	—0.877 **	—0.118	—0.599	—0.627

* Significant at 5% level.

** Significant at 1% level.

THE AMOUNT OF NUTRIENTS IN AVERAGE TREES

As the trees become older, increasing amounts of nutrients are incorporated within them (Table III). This increase occurs prima-

TABLE III

The mineral content (g) of average trees									
	Tree age yrs.								
	6	24	27	32	38	42	46	53	55
<i>Sodium</i>									
Leaves	0.001	0.14	0.08	0.05	0.07	0.17	0.60	0.41	1.13
Live branches	0.004	0.17	0.27	0.16	0.51	0.52	3.50	3.90	3.19
Bole	0.004	0.15	0.52	0.22	0.75	0.79	2.21	2.03	2.19
Total	0.009	0.46	0.87	0.43	1.33	1.48	6.31	6.34	6.51
Roots	0.004	0.26	—	—	—	4.73	—	—	4.69
Dead branches	0.001	0.02	0.04	0.04	0.18	0.31	0.41	0.27	0.18
<i>Potassium</i>									
Leaves	0.16	5.6	9.8	5.8	7.3	12.3	32.6	39.2	49.2
Live branches	0.10	4.3	7.1	3.7	9.8	9.7	31.1	58.6	52.7
Bole	0.18	5.8	18.4	7.3	21.7	22.8	64.9	69.6	73.8
Total	0.44	15.7	35.3	16.8	38.8	44.8	128.6	167.4	175.7
Roots	0.03	3.1	—	—	—	13.8	—	—	52.0
Dead branches	< 0.01	0.1	0.2	0.3	0.9	1.8	1.1	1.8	0.5
<i>Calcium</i>									
Leaves	0.16	6.5	9.4	4.8	7.0	14.1	42.5	30.6	48.2
Live branches	0.26	13.9	16.3	12.9	43.4	27.4	104.2	177.1	204.0
Bole	0.36	26.4	56.2	28.0	97.8	118.1	324.9	289.7	312.8
Total	0.78	46.8	81.9	45.7	148.2	159.6	471.6	497.4	565.0
Roots	0.10	15.9	—	—	—	59.4	—	—	175.5
Dead branches	0.03	1.8	1.2	3.0	14.5	16.1	16.0	11.1	10.8
<i>Magnesium</i>									
Leaves	0.04	1.1	1.5	0.8	1.2	2.4	5.4	6.3	8.5
Live branches	0.02	1.1	1.7	1.1	3.5	3.3	12.1	14.6	14.8
Bole	0.02	2.5	5.7	3.3	8.9	8.8	26.0	25.9	30.1
Total	0.08	4.7	8.9	5.2	13.6	14.5	43.5	46.8	53.4
Roots	0.01	0.9	—	—	—	7.4	—	—	16.0
Dead branches	< 0.01	0.1	0.1	0.2	0.5	0.8	0.7	0.6	0.4
<i>Phosphorus</i>									
Leaves	0.03	0.5	1.1	0.6	0.8	1.4	3.5	3.5	4.6
Live branches	0.03	1.1	2.1	0.8	2.1	3.0	6.9	18.1	13.3
Bole	0.04	1.2	4.1	1.4	3.4	4.6	10.4	12.0	12.4
Total	0.10	2.8	7.3	2.8	6.3	9.0	20.8	33.6	30.3
Roots	0.01	1.0	—	—	—	3.0	—	—	8.3
Dead branches	< 0.01	< 0.1	< 0.1	0.1	0.3	0.4	0.2	0.5	0.2
<i>Nitrogen</i>									
Leaves	0.29	9.4	17.7	11.5	14.4	22.7	64.9	59.0	88.5
Live branches	0.29	13.7	24.6	12.9	29.6	38.1	67.9	243.5	190.6
Bole	0.26	14.7	37.5	16.4	48.4	55.0	138.4	161.9	165.2
Total	0.84	37.8	79.8	40.8	92.4	115.8	271.2	464.4	444.3
Roots	0.11	17.9	—	—	—	73.4	—	—	173.1
Dead branches	0.01	1.5	0.6	1.5	7.7	7.7	4.9	7.5	3.9

rily as a result of the large increase in dry weight for the percentages of nutrients in the boles, for instance, decrease with greater age. After 55 years growth, the surviving birch trees on average contain 11.4 g of sodium, 228 g of potassium, 751 g of calcium, 70 g of magnesium, 39 g of phosphorus and 621 g of nitrogen. These elements are not distributed equally between the component parts of the trees and the distribution depends on both the dry weight and the average percentages of the elements in the tree parts. Thus, the canopy of the oldest birch tree is only a fifth of the dry weight of the living shoot but contains about two thirds

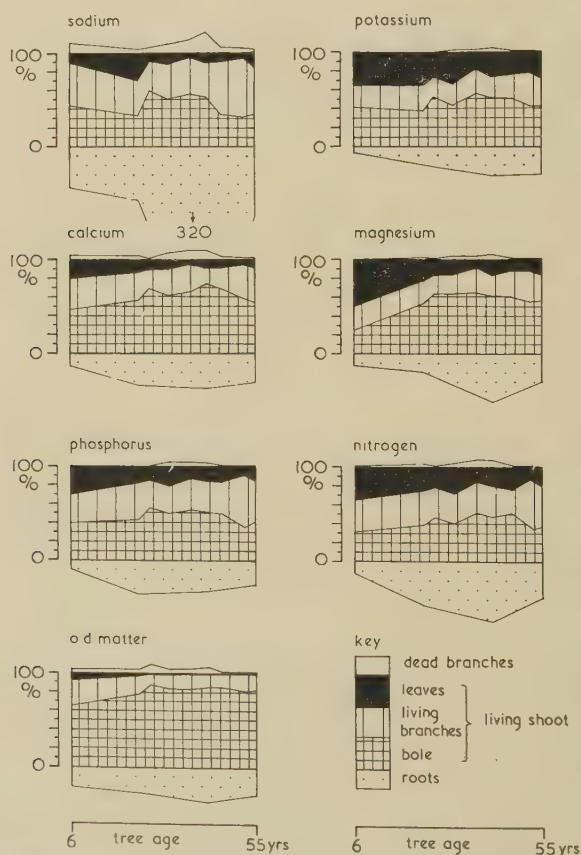


Fig. 2. The distribution of dry matter, sodium, potassium, calcium, magnesium, phosphorus, and nitrogen in birch trees of different ages. Expressed as percentages of the weight of the living shoot.

of the weights of nutrients in the shoot. In particular, the leaves contain relatively large amounts of nutrients for their weight for although they only represent 2 per cent of the dry weight, they contain 5 to 30 per cent of the sodium, 19 to 36 per cent of the potassium, 5 to 21 per cent of the calcium, 9 to 50 per cent of the magnesium, 10 to 30 per cent of the phosphorus and 13 to 35 per cent of the nitrogen in the living shoot. Similarly, the branches contain about 18 per cent of the dry matter, but in most cases, higher proportions of each of the elements, *i.e.* 31 to 62 per cent of the sodium, 20 to 35 per cent of the potassium, 17 to 36 per cent of the calcium, 19 to 31 per cent of the magnesium, 29 to 54 per cent of the phosphorus and 25 to 52 per cent of the nitrogen. In contrast, although the bole represents about 80 per cent of the dry weight of the living shoot, it only contains 32 to 60 per cent of the sodium, 37 to 56 per cent of the potassium, 46 to 74 per cent of the calcium, 25 to 65 per cent of the magnesium, 36 to 56 per cent of the phosphorus and 31 to 52 per cent of the nitrogen.

On the whole, the tree canopy contains a greater weight of nutrients than the bole and the bole more than the root system, except for sodium and nitrogen, which occur in relatively large amounts in the tree roots. Although the total nutrient content of all the different parts of the trees tends to increase with age, the leaves contain a decreasing proportion of the nutrients since, because of the annual leaf fall, the build-up of leaf material per tree is not so rapid as that of branch, bole or root matter (Fig. 2).

THE AMOUNT OF NUTRIENTS IN TREE STANDS

The amounts of nutrients contained in all the living trees comprising each woodland have been estimated by multiplying the weights of nutrients in the average trees by the number of trees per hectare (Table IV). The increase in the total quantities of the six elements with greater tree age closely parallels changes in dry weight. For example, the amounts of the nutrients in the tree boles become steadily greater as the woodlands become older, but the nutrient content of the tree leaves increases very rapidly between 6 to 24 years of age as a leaf mass develops but afterwards only increases slightly since the leaf weight within the woodland canopy becomes relatively constant up to 55 years of age. From about 32

TABLE IV

The mineral content of tree stands (kg/ha)									
	Tree age yrs.								
	6	24	27	32	38	42	46	53	55
<i>Sodium</i>									
Leaves	0.01	0.7	0.2	0.2	0.1	0.2	0.4	0.4	1.0
Live branches	0.04	0.8	0.7	0.7	0.8	0.7	2.6	4.0	2.8
Boles	0.04	0.7	1.3	0.9	1.1	1.1	1.6	2.1	1.9
Total	0.09	2.2	2.2	1.8	2.0	2.0	4.6	6.5	5.7
Roots	0.04	1.3	—	—	—	6.3	—	—	4.1
Dead branches	0.01	0.1	0.1	0.2	0.3	0.4	0.3	0.3	0.2
<i>Potassium</i>									
Leaves	1.6	28	24	25	11	16	24	40	43
Live branches	1.0	21	18	15	15	13	23	60	46
Boles	1.8	29	46	31	32	31	48	71	65
Total	4.4	78	88	71	58	60	95	171	154
Roots	0.3	15	—	—	—	18	—	—	46
Dead branches	< 0.1	< 1	< 1	1	1	2	1	2	< 1
<i>Calcium</i>									
Leaves	1.6	32	23	20	10	19	31	31	42
Live branches	2.7	69	40	54	65	37	77	181	180
Boles	3.8	132	139	118	147	158	240	295	275
Total	8.1	233	202	192	222	214	348	507	497
Roots	1.1	79	—	—	—	80	—	—	154
Dead branches	0.3	9	3	13	21	22	12	11	10
<i>Magnesium</i>									
Leaves	0.4	5	4	4	2	3	4	6	7
Live branches	0.2	6	4	5	5	4	9	15	13
Boles	0.3	13	14	14	13	12	19	26	26
Total	0.9	24	22	23	20	19	32	47	46
Roots	0.1	4	—	—	—	10	—	—	14
Dead branches	< 0.1	< 1	< 1	1	1	1	1	1	< 1
<i>Phosphorus</i>									
Leaves	0.3	3	3	3	1	2	3	4	4
Live branches	0.3	5	5	3	3	4	5	18	12
Boles	0.4	6	10	6	5	6	8	12	11
Total	1.0	14	18	12	9	12	16	34	27
Roots	0.1	5	—	—	—	4	—	—	7
Dead branches	< 0.1	< 1	< 1	< 1	< 1	< 1	< 1	1	< 1
<i>Nitrogen</i>									
Leaves	3.1	47	44	49	22	30	48	60	78
Live branches	3.1	68	61	54	44	51	50	248	168
Boles	2.7	73	93	69	73	74	102	165	145
Total	8.9	188	198	172	139	155	200	473	391
Roots	1.2	89	—	—	—	98	—	—	152
Dead branches	0.1	7	2	6	12	10	4	8	3

to 42 years the total nutrient content of the living trees does not vary greatly and may even decline slightly because a temporary reduction in leaf weight at this stage compensates for any increase in the accumulation of nutrients within the living tree boles. After 42 years of age there is a renewed accumulation of nutrients within the stand as a whole so that the trees of the oldest woodland contain, as kilograms per hectare, 10 of sodium, 200 of potassium, 661 of calcium, 60 of magnesium, 34 of phosphorus and 546 of nitrogen after 55 years. The average annual incorporation of sodium, potassium, calcium, magnesium, phosphorus and nitrogen within the trees, throughout the fifty-five years, amounts to 0.2, 3.6, 12.0, 1.1, 0.6 and 9.9 kilograms per hectare respectively but this only applies to the summer period for in autumn when the leaves have been shed the values would be reduced.

THE NUTRIENT CYCLE

The nutrient content of the tree stock does not represent the gross uptake of nutrients by the tree since some trees will have died and some of the nutrients taken up by the surviving trees will have been returned to the soil in various ways. Consequently, the gross uptake of nutrients by the trees has been calculated from the estimates of total dry-matter production. During the period from 6 to 55 years when the canopies have closed and dry-matter production is high, the gross annual uptake of nutrients by the trees, excluding roots, is relatively constant averaging 0.6, 28, 44, 5.6, 4.1 and 56 kilograms per hectare for sodium, potassium, calcium, magnesium, phosphorus and nitrogen respectively. Leaf fall returns to the soil about half of the nutrient uptake and this is further augmented by branches falling from the trees and the death of weaker trees to give an average annual return, as kilograms per hectare, of 0.5 for sodium, 25 for potassium, 34 for calcium, 4.7 for magnesium, 3.6 for phosphorus and 48 for nitrogen. These values are only slightly less than the annual uptake of the various elements and the difference between the two sets of figures represents the annual increase of nutrients within the tree mass during the period. Decomposition of dead tree material occurs fairly rapidly on the base rich peat and the fallen birch leaves rarely persist for more than a year so that the annual return of nutrients in the litter fall

is approximately equal to the amount of nutrients released yearly by decomposition of organic matter derived from the trees. The estimates of nutrient return to the soil will be underestimates for they do not include nutrients washed out of the birch canopies by precipitation (Madgwick and Ovington⁵) and the release of nutrients by the annual decay of root material from living trees. Comparable nutrient cycles may occur between the ground flora and the soil but this is not likely to be of great importance in the birch stands for the ground vegetation in such dense woodlands is absent or poorly developed.

DISCUSSION

Holme Fen is located in the Fenland region, one of the most intensively cultivated areas of Britain characterised by high agricultural productivity once the peat soils are effectively drained. Consequently, there are only small areas of semi-natural vegetation left in the Fens but Kassas^{2 3} has described the natural tree and bush colonization of nearby Chippenham Fen and gives the dry weight of the *Molinia-Phragmites* community there as approx. 3,550 kilograms per hectare. The birch stands would attain this weight about 7 to 8 years after colonisation but the average nutrient content of the trees is probably much less than that of the herbaceous flora so that it would take about 20 years before the nutrient content of the trees equalled that of the herbaceous vegetation type. Compared with other tree species (Ovington^{7 8}), the birch stands do not contain very large amounts of plant nutrients but the total quantity of nutrients contained in the trees after fifty-five years does represent a significant retention of nutrients. The amounts retained are relatively small when compared with the gross uptake by the trees and, of the total uptake, about 77 per cent of the sodium, 87 per cent of the potassium, 74 per cent of the calcium, 81 per cent of the magnesium, 85 per cent of the phosphorus and 84 per cent of the nitrogen have been returned to the soil after 55 years. The woodland community is therefore characterised by a large interchange of nutrients between the trees and soil.

With the need for more intensive forest management increased attention is being paid to the possibilities of applying artificial fertilizers to replace the nutrients removed when a tree crop is

harvested. Viro¹⁵ and Tamm^{13 14} have demonstrated that the application of fertilizers to birch woodlands may increase the nutrient content of the leaves and improve tree growth. The fertilizer-application experiments of Tamm were, however, located on peats much poorer in mineral nutrients than the fen peat and the nutrient content of the birch leaves at Holme Fen tends to be greater than those from the unfertilized plots in Tamm's experiment. The nutrient content of the leaves of the Holme Fen birch trees is comparable to that of birch growing on base rich mineral soil in the same general climatic region (Ovington⁶). The potassium, magnesium and calcium values for the leaf material lie within Ingestad's¹ 90 per cent of control growth range and phosphorus and nitrogen above the 50 per cent level for seedlings grown in nutrient solutions. It seems unlikely that nutrient deficiency of the soil is limiting the growth of the birch on the fen peat and the nutrient uptake must be approaching the maximum for the species in that region. Waterlogging as a result of failure to maintain the drains may have reduced productivity and consequently total nutrient uptake at certain stages of growth.

In recent years increased interest has been aroused in determining the effects of nutrient uptake by forest stands on soil-forming processes (Remezov¹¹, Rennie¹²). There is no evidence that the establishment and growth of the birch stands has caused any reduction of soil fertility but such effects would be long term and would need to be measured in terms of the breakdown of the peat and the general circulation of nutrients within the woodland ecosystem.

SUMMARY

Sample trees up to 55 years of age from nine natural woodlands of birch at Holme Fen Nature Reserve were analysed for sodium, potassium, calcium, magnesium, phosphorus, and nitrogen. Data are given of the amounts of these elements in the leaves, branches, boles, and in some cases of roots for the sample trees and the stock of the tree stands. The average annual uptake of nutrients and the return of nutrients to the soil as the woodlands mature are estimated.

REFERENCES

- 1 Ingestad, T., Studies on the nutrition of forest tree seedlings I. Mineral nutrition of birch. *Physiol. Plantarum* **10**, 418 (1957).
- 2 Kassas, M., Studies in the ecology of Chippenham Fen III. The Forty Acre Wood. *J. Ecol.* **40**, 50 (1952).
- 3 Kassas, M., Studies in the ecology of Chippenham Fen IV. Tree and bush colonization in South Chippenham Fen. *J. Ecol.* **62**, 50 (1952).
- 4 Leyton, L., Needle composition in relation to the growth and nutrition of Japanese larch. *In: Plant Analysis and Fertilizer Problems*. I.R.H.O. Paris, 143 (1957).
- 5 Madgwick, H. A. I. and Ovington, J. D., The chemical composition of precipitation in adjacent forest and open plots. *Forestry* (In press) (1958).
- 6 Ovington, J. D., The composition of tree leaves. *Forestry* **29**, 22 (1956).
- 7 Ovington, J. D., The volatile matter, organic carbon and nitrogen contents of tree species grown in close stands. *New Phytologist* **56**, 1 (1957).
- 8 Ovington, J. D. and Madgwick, H. A. I., The sodium, potassium and phosphorus contents of tree species grown in close stands. *New Phytologist* **58**, 273 (1958).
- 9 Ovington, J. D. and Madgwick, H. A. I., The growth and composition of natural stands of birch I. Dry matter production. *Plant and Soil* **10**, 271-283 (1959).
- 10 Piper, C. S., *Soil and Plant Analysis*. Monograph from the Waite Agricultural Research Institute (1950).
- 11 Remezov, N. P., The role of the biological circulation of the elements in soils formation under the forests. *Sixième Congr. de la Sci. Sol 6e Congr. Paris 1956 E*, 269 (1956).
- 12 Rennie, P. J., The uptake of nutrients by mature forest growth. *Plant and Soil* **7**, 49 (1955).
- 13 Tamm, C. O., Studies on Forest Nutrition. 3. The effects of supply of plant nutrients to a forest stand on a poor site. *Medd. Statens Skogsforskn. Inst.* **46** (3), 34 pp. (1956).
- 14 Tamm, C. O., Studies on Forest Nutrition. 4. The effects of supply of potassium and phosphorus to a poor stand on drained peat. *Medd. Statens Skogsforskn. Inst.* **46** (7), 27 pp. (1956).
- 15 Viro, P. J., Fertilization trials on forest soil. *Comm. Inst. Forest. Fenniae* **37**, 1-23 (1950).
- 16 Wright, T. W. and Will, G. M., The nutrient content of Scots and Corsican Pines growing on sand dunes. *Forestry* **31**, 13 (1958).

②
U Haw &